Universidade do Minho
Escola de Engenharia

Margarida Isabel de Barros Coelho Martins

Insights into *Candida* world :the extracellular milieu

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Insights into *Candida* world :the extracellular milieu

Doutoramento em Engenharia Biomedica

Trabalho efectuado sob a orientação da
Professora Doutora Domingas do Rosario Oliveira
e da
Professora Doutora Mariana Henriques

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DECLARAÇÃO

Nome: Margarida Isabel de Barros Coelho Martins

Endereço Electrónico: martins.margarida@gmail.com

Telefone: +351 934640763

Número do Bilhete de Identidade: 11001960

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Orientadores:

Professora Doutora Domingas do Rosário Oliveira

Professora Doutora Mariana Henriques

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Para o avô João e o avô Martins,
que tanto gostariam de ver esta obra.

"Deus quer, o Homem sonha, a obra nasce ..."

"God wills, Man dreams, the work is born ..."

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Outline of the thesis

The present thesis reports the works performed at (i) Rosário Oliveira's lab at IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal, (ii) Manuel Coimbra's lab at Departamento de Química, Universidade de Aveiro, Aveiro, Portugal, (iii) José Lopez-Ribot's lab at Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, Texas, USA, and (iv) Fernando Rodrigues's lab at Life and Health Sciences Research Institute (ICVS), School of Health Sciences, Universidade do Minho, Braga, Portugal, to complete my Ph.D. work plan.

This thesis is organized into five chapters. The first chapter briefly reviews relevant aspects of Candida species biology and summarizes the state-of-the-art knowledge on *Candida* species extracellular molecules, other than proteins. Chapters 2 and 3 report the studies performed on the analysis of compounds released by *Candida* species into the extracellular medium and on the elucidation of the interactions of these molecules with virulence traits of *Candida* species. The research strategy consisted of selecting extracellular DNA (Chapter 2) and a set of alcohol compounds (Chapter 3), based on previous investigations on the fungal field. The fourth chapter focuses on the evaluation of *Candida* species in a clinical setting. Finally, general conclusions are presented in Chapter 5, unifying the published and submitted data and suggesting clues for future work.

Abstract

Over the last years fungi have emerged as a major cause of human disease. *Candida albicans* is the most common cause of opportunistic mycoses, albeit Non-*Candida albicans Candida* (NCAC) species, namely *Candida dubliniensis*, *Candida glabrata*, *Candida krusei, Candida parapsilosis*, and *Candida tropicalis*, are emerging as pathogens. *Candida* species factors that might influence the pathogenesis of infection include the ability to: undergo a reversible conversion between yeast and filaments (only for *C. albicans* and *C. dubliniensis*), form biofilms, and secrete proteins and chemicals, such as *E,E*-farnesol (farnesol), into the extracellular medium. This work aimed to bring insights into *Candida* species world, following two main routes: first, the analyses of compounds released by *Candida* species into the extracellular medium focusing on extracellular DNA (eDNA) and alcohol compounds, and second, the identification of *Candida* species in clinical samples.

Concerning eDNA, here we show that it is a component of *C. albicans* biofilm extracellular matrix (ECM). In addition, based on the degradation of eDNA by deoxyribonuclease (DNase) and addition of exogenous DNA at different stages of biofilm development, it was possible to define that eDNA contributes to the maintenance and stability of *C. albicans* mature biofilms. Furthermore, the study of the impact of the combined use of DNase and antifungals against *C. albicans* biofilms revealed that DNase increases the effectiveness of the antifungal agents amphotericin B and caspofungin. Our results present evidence that eDNA is a key element of the ECM in *C. albicans*, contributing to biofilm integrity and antifungal resistance.

With respect to extracellular alcohols, the results presented herein show that *C. albicans* extracellular alcohol farnesol impairs *C. glabrata*, *C. krusei*, and *C. tropicalis* planktonic growth by interfering with cell viability and/or cell cycle, depending on the species. In addition, headspace-solid-phase microextraction and gas chromatography-mass spectrometry were used for profiling *Candida* extracellular alcohols. Those procedures allowed the identification and quantification of isoamyl alcohol, 2-phenylethanol (phenylethanol), 1-dodecanol, *E*-nerolidol (nerolidol), and farnesol in *C. albicans* and *C. dubliniensis* planktonic and biofilm cells supernatants. With the exception of nerolidol, these compounds were also identified in *C. parapsilosis* and *C. tropicalis* planktonic supernatants. Based on the quantifications performed in this work, the effect of the exogenous addition of commercial formulations of the alcohols was tested in vitro and in vivo.

In vitro, physiological levels of these alcohols were capable of inhibiting *C. albicans* and *C. dubliniensis* yeast to filament conversion, under filamentation inducing conditions, when used alone or in combination (simulating a 96-h culture supernatant). Furthermore, the evaluation of the impact of the individual addition of the extracellular alcohols against *Candida* species biofilm cells mitochondrial activity and biofilm biomass showed that these compounds differentially affect biofilm development. Of note, physiological levels of isoamyl alcohol, phenylethanol, and nerolidol interfered with *C. parapsilosis* and *C. tropicalis* biofilm development. In vivo, using the well established murine model of hematogenously disseminated candidiasis, it was shown that a solution simulating the composition of alcohols present in a *C. albicans* culture supernatant is able to increase mice survival, decreasing fungal burden, and filamentation in the kidney. Overall, these results show that extracellular compounds produced by *Candida* species regulate their virulence traits.

Finally, we evaluated the oral *Candida* carriage in patients attending a dental clinic in Braga, Portugal. This study showed that although *C. albicans* is the *Candida* species most frequently isolated from the oral cavity in this population, NCAC species also contribute to the fungal burden. Notably, the majority of these NCAC species was co-isolated with *C. albicans*, evidencing the complex control of *Candida* species diversity and distribution in a natural environment.

Taken together, these studies represent a very important contribution to our understanding of the composition of the extracellular milieu of *Candida* species and its relationship with the regulation of *Candida* biology, and open the possibility of the development of new treatment and/ or diagnostic strategies to combat candidiasis.

Resumo

Ao longo dos últimos anos os fungos têm emergido como uma das principais causas de doenças humanas. *Candida albicans* constitui a principal causa de micoses oportunistas, embora outras espécies de *Candida* não *albicans* (CNA), como *Candida dubliniensis*, *Candida glabrata*, *Candida krusei, Candida parapsilosis* e *Candida tropicalis* estejam a emergir como patogénicos. São vários os factores que podem influenciar a patogénese, nomeadamente, a capacidade de transitar reversivelmente entre as formas de levedura e filamento (especificamente *C. albicans* e *C. dubliniensis*), a capacidade de formar biofilme e ainda a de libertar proteínas e outras classes de compostos, como o *E,E*-farnesol (farnesol), no meio extracelular. Esta tese teve como objectivo principal o estudo do universo das espécies de *Candida* seguindo, nesse sentido, duas linhas de investigação. Primeiro, efectuando a avaliação dos compostos libertados por espécies de *Candida* para o meio extracelular, especificamente DNA extracelular (DNAe) e compostos alcoólicos. Segundo, procedendo à identificação de espécies de *Candida* em amostras clínicas.

Relativamente aos estudos que visaram o DNAe, os nossos resultados mostraram que este é um componente da matriz extracelular de biofilmes formados por *C. albicans*. Adicionalmente, com base na degradação do DNAe pela enzima desoxiribonuclease e na adição de DNA exógeno em diferentes fases de desenvolvimento do biofilme, foi possível estabelecer que o DNAe contribui para a manutenção e estabilidade de biofilmes maduros de *C. albicans*. Complementarmente, o estudo do efeito da combinação da desoxiribonuclease com agentes antifúngicos em biofilmes de *C. albicans* revelou que a desoxiribonuclease aumenta a eficácia dos agentes antifúngicos anfotericina B e caspofungina. Os nossos resultados evidenciam que o DNAe é um elemento chave da matriz extracelular de biofilmes de *C. albicans* que contribui para a sua integridade e resistência a agentes antifúngicos.

Em relação aos álcoois extracelulares, os resultados apresentados nesta tese revelaram que o farnesol, álcool extracelular produzido por *C. albicans*, afecta negativamente o crescimento de células planctónicas de *C. glabrata*, *C. krusei* e *C. tropicalis*, por interferir com a viabilidade e/ou ciclo celular, dependendo da espécie em estudo. Adicionalmente, a técnica de microextracção em fase sólida, em modo de espaço de cabeça acoplada a cromatografia de gás-espectrometria de massa foi utilizada para determinar o perfil dos álcoois extracelulares de espécies de *Candida*. Esta análise permitiu a identificação e quantificação dos álcoois: álcool isoamílico,

2-feniletanol (feniletanol), 1-dodecanol, *E*-nerolidol (nerolidol) e farnesol em sobrenadantes obtidos de culturas de *C. albicans* e *C. dubliniensis* na forma planctónica e em biofilme. Estes compostos, à excepção do nerolidol, foram também identificados em culturas planctónicas de *C. parapsilosis* e *C. tropicalis*. De seguida, tendo por base as quantificações dos compostos determinadas no decurso deste trabalho, foram efectuados ensaios no sentido de avaliar o efeito destes compostos *in vitro* e *in vivo*. Por um lado, nos ensaios *in vitro*, verificou-se que concentrações fisiológicas destes compostos inibem a transição morfológica de levedura para filamento de *C. albicans* e *C. dubliniensis*, sob condições que promovem a filamentação. Este efeito foi observado quando os álcoois foram adicionados ao meio de cultura individualmente ou em combinação, simulando um sobrenadante de 96 h. De seguida, a avaliação do efeito da adição de álcoois extracelulares na actividade mitocondrial e biomassa de biofilmes de espécies de *Candida* mostrou que estes compostos interferem com o desenvolvimento do biofilme. De notar que observámos que concentrações fisiológicas de álcool isoamílico, de feniletanol e nerolidol regulam a formação de biofilme de *C. parapsilosis* e *C. tropicalis*. Por outro lado, para os ensaios *in vivo* foi utilizado um modelo de murino de candidiase já estabelecido e caracterizado. Os resultados revelaram que a administração intraperitoneal de uma solução mimetizando um sobrenadante de *C. albicans* relativamente à sua composição em álcoois, conduz a um aumento da sobrevivência dos ratinhos, acompanhado pela diminuição da carga fúngica e da filamentação a nível renal. De uma forma geral, estes resultados mostram que moléculas extracelulares produzidas por espécies de *Candida* são capazes de regular os seus factores de virulência.

Finalmente, avaliámos a colonização oral por espécies de *Candida* numa população de pacientes de uma clínica dentária em Braga, Portugal. Este estudo mostrou que nesta população, embora *C. albicans* tenha sido a espécie de *Candida* mais frequente, as espécies CNA também contribuem para a carga fúngica na cavidade oral. De notar que a maioria das CNA foi co-isolada com *C. albicans*, evidenciando a complexidade do controlo da diversidade e distribuição de espécies de *Candida* num ambiente natural.

Em conclusão, os resultados apresentados nesta tese contribuem significativamente para a elucidação da composição do meio extracelular de espécies de *Candida* e da sua relação com a regulação da biologia das próprias espécies de *Candida*, abrindo novas perspectivas no sentido de desenvolver novos tratamentos e/ou terapias de diagnóstico para combater a candidiase.

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Abbreviations and acronyms

Abbreviation/ Acronym: Term
µg: microgram
µm: micrometer
µM: micromolar
A_{260}: Absorbance at 260 nm
A_{280}: Absorbance at 280 nm
A_{490}: Absorbance at 490 nm
A_{550}: Absorbance at 550 nm
A_{570}: Absorbance at 570 nm
A_{640}: Absorbance at 640 nm
ATCC: American Type Culture Collection
bp: base pairs
C. albicans: *Candida albicans*
C. dubliniensis: *Candida dubliniensis*
C. famata: *Candida famata*
C. glabrata: *Candida glabrata*
C. guilliermondii: *Candida guilliermondii*
C. krusei: *Candida krusei*
C. parapsilosis: *Candida parapsilosis*
C. tropicalis: *Candida tropicalis*
cAMP: cyclic adenosine monophosphate
CBS: Centraalbureau voor Schimmelcultures
CECT: Colección Española de Cultivos
CFU: Colony Forming Unit
CW/DVB: Cross-linked Carbowax-divinylbenzene
DNA: Desoxiribonucleic acid
DNase: Deoxyribonuclease I
dNTP: deoxyribonucleotide triphosphate
Dodecanol: 1-Dodecanol
DOH: 1-Dodecanol
e.g.: (*exempli gratia*) for example
ECM: Extracellular matrix
eDNA: extracellular DNA
et al.: (*et alii*) and others
eV: electron volt
Farnesol: *E,E*-Farnesol
Fig.: Figure
FOH: *E,E*-Farnesol
g: gram
GC-MS: Gas Chromatography-Mass Spectrometry
h: hour
HPLC: High Performance Liquid Chromatography
HS-SPME: Headspace-Solid-Phase Microextraction
i.e.: (*id est*) that is
i.p. : intraperitoneally
IAA: Isoamyl alcohol
Kb: Kilobase

kg: kilogram
l: liter
LD: Lethal Dose
ln: natural logarithm
log_{10}: logarithm with base 10
M: Molar
mg: milligram
min: minute
ml: mililiter
mM: milimolar
mRNA: messenger ribonucleic acid
mW: miliwatt
n:number
NCAC: Non–*Candida albicans Candida*
Nerolidol: *E*-Nerolidol
ng: nanogram
nM: nanomolar
NOH: *E*-Nerolidol
P: Probability
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
pH: potential hydrogen
Phenylethanol: 2-Phenylethanol
POH: 2-Phenylethanol
RNase: Ribonuclease
rpm: revolutions per minute
RPMI: Roswell Park Memorial Institute 1640
S. cerevisiae: *Saccharomyces cerevisiae*
s: second
SD: Standard Deviation
SE: Silicone Elastomer
SEM: Standard Error of the Mean
SMIC50: Sessile Minimum Inhibitory Concentrations determined at 50% decrease in absorbance at 490 nm
SMIC80: Sessile Minimum Inhibitory Concentrations determined at 80% decrease in absorbance at 490 nm
TBE: Tris-Borate-EDTA
TE: Tris-EDTA
TLR9: Toll-Like Receptor 9
Triton X-100: octylphenol ethylene oxide condensate
t-test: Student's t test
Tween 20: Polysorbate 20
U: units
UV: Ultra violet
vs: (*versus*) against
W: watt
XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide
YNB: Yeast Nitrogen Base
YPD: Yeast extract-Peptone-Dextrose

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CHAPTER 1 General Introduction

Starting in the early 1980s, fungal diseases began to constitute a major public health problem. The observed increase in opportunistic fungal infections has parallel with the expansion in populations with metabolic and immunosuppressive diseases, and mucosal or cutaneous barrier disruption, as a consequence of the advances in medical practices [1, 2]. Overall, *Candida* species are now recognized as major agents of opportunistic fungal infections worldwide. Although for many years *Candida* infections were synonymous of disease caused by *Candida albicans*, the recent surveillance studies indicate that Non-*Candida albicans Candida* (NCAC) species, particularly, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei, Candida parapsilosis*, and *Candida tropicalis* are emerging as pathogens [1, 3, 4]. This species distribution shift has been attributed to NCAC species intrinsic or acquired resistance to antifungal agents, namely fluconazole [3]. The recognition of *Candida* infections as a life-threatening problem associated with high mortality rates and costs, due to increased length of patients hospitalization, antifungals acquisition, ineffective treatment, and antifungals side effects [5], probably are responsible for the increasing interest in the study of *Candida* and candidiasis.

While fundamental researchers focus on *Candida* species biology, clinicians are concerned with the invasive form of the disease [6]. In fact, *Candida* species make part of the commensal flora of healthy individuals [7], as stated by Professor Carol Kumamoto, "*C. albicans* has an identity crisis; it thinks it's a part of the human body" (reported in [8]). However, when the host immune system is impaired for example by immunosuppressive treatments, or the competing flora is altered by antibiotics or hormonal therapies, *Candida* species can overwhelm the protective host defense mechanisms and become pathogenic, thus causing disease. The spectrum of clinical manifestations ranges from skin and mucous membrane lesions, such as the observed in the oral cavity, to life-threatening invasive infections, arising from bloodstream invasion and hematogenously spread of the *Candida* species [9]. To fulfill the clinicians' demands of new diagnostic tools, efficient treatments for invasive candidiasis [10], and to direct the research into the patients needs, it is important to establish a strict cooperation between fundamental researchers and clinicians for the understanding of (i) the distribution and epidemiology of *Candida* species in specific niches, and (ii) *Candida* biology, including the fungal specific attributes and strategies associated with pathogenesis, ie, *Candida* virulence factors.

Although fungal virulence is thought to be multifactorial, over the years some virulence factors have been highlighted in *Candida* species (reviewed in [11-16]), with special focus on *C. albicans*. The fact that for many years NCAC species were considered benign for the human host may have diverted researchers from studying these species, delaying the development of molecular tools and animal models. Actually, to define the genes responsible for virulence it is essential to show that the null mutants have attenuated virulence and that the addition of the gene back to the mutant restores the virulence [17]. Thus, to date, the research on NCAC species virulence factors seems to be driven by the evaluation of factors shared by *C. albicans* and NCAC species, and not on the investigation of factors that distinguish them.

CANDIDA SPECIES MORPHOGENESIS

From the observation that during infection *C. albicans* grows in both yeast and filamentous form (Fig.1.1 *A*) emerged one of the branches of *Candida* biology – morphogenesis -. Not only in vivo, but also in vitro *C. albicans* can undergo a reversible conversion from yeast (Fig.1.1 *B*) to hyphae (Fig.1.1 *D*), passing through an intermediate form, the pseudohyphae (Fig.1.1 *C*) [18]. Notably, among NCAC species, the ability to grow in yeast and hyphae forms is only shared by *C. dubliniensis* [15]. In fact, although all the NCAC species are able to form yeast, and the majority can form pseudohyphae, only *C. dubliniensis* can form true hyphae [19]. However, *C. dubliniensis* seems to have less ability to filament in vitro and in vitro [20].

The *C. albicans* morphological transition from yeast to hyphae depends on the environmental conditions in which *C. albicans* is growing. Several environmental factors such as serum, *N*-acetylglucosamine, neutral pH, starvation, temperature above 35°C, cell density

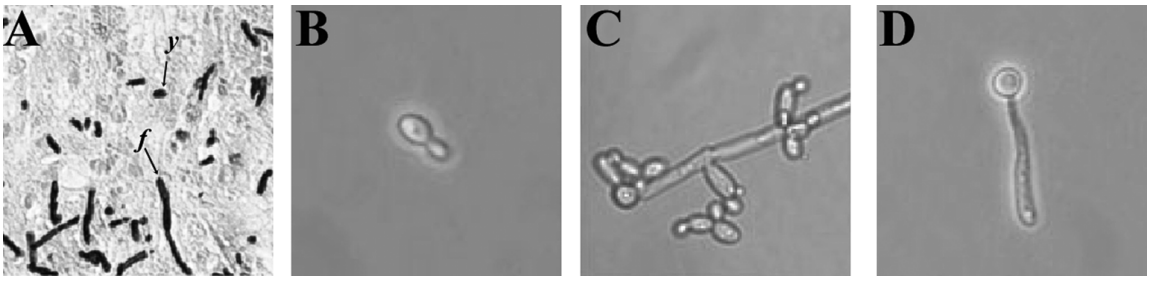

Fig.1.1. Common growth forms of *C. albicans*. Microscopy analysis of (*A*) histopathological analysis of a kidney from a mouse infected with C. albicans, exhibiting both yeast (y) and filamentous (f) forms, and in vitro cultured cells under experimental conditions that promote (*B*) yeast, pseudohyphae (*B*), and hyphae (*C*) forms growth.

below 10^6 cells/ml have been described as effectors of the conversion from yeast to hyphae through different signaling pathways (reviewed in [21, 22]). Of note, two additional forms adopted by *C. albicans*, the opaque and chlamydospores forms, whose function has not been deeply investigated [23], can also be regulated by environmental factors [24, 25].

The association between *C. albicans* morphogenesis and virulence arises from several lines of evidences suggesting that yeast and hyphae forms are required for the pathogenecity, being proposed that while yeast cells are more suitable for dissemination, hyphae may be essential for infection, colonization, and biofilm structure (reviewed in [26, 27]). However, it is not clear whether morphogenesis, per se, is a virulence factor, or it is its co-regulation with other virulence traits that promotes pathogenecity [28].

CANDIDA SPECIES BIOFILM FORMING ABILITY

The analysis of the predisposing factors underlying NCAC species infections [29] indicates that among other factors, the *C. glabrata* and *C. parapsilosis* candidaemia correlates with intravenous catheters and prosthetic heart valves, respectively [29]. This suggests that the ability of NCAC species to adhere to a biomaterial represents the first stage of the infection process. In fact, as reviewed by Ramage et al. [30], virtually all implanted devices can be colonized by endogenous and/or exogenous *Candida* species, constituting this the first of four steps that end in the formation of (mature) biofilms (Fig.1.2). In a genetically controlled process, the cell adhesion and

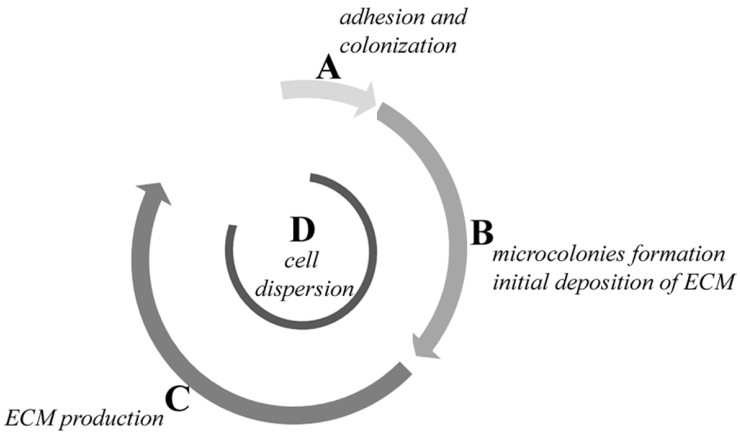

Fig.1.2. Schematic diagram of *C. albicans* biofilm life cycle. The four main phases of biofilm development, (***A***) early, (***B***) intermediate, (***C***) maturation, and (***D***) dispersion are indicated as well as the key processes occurring at each phase, according to Chandra *et al.* [31] and Uppuluri *et al.* [32]. ECM means extracellular matrix.

colonization (Fig.1.2 *A*, early phase) is followed by cell division and proliferation with formation of microcolonies and emergence of self produced extracellular material, the extracellular matrix (ECM) (Fig.1.2 *B*, intermediate phase). In the maturation phase, there is an increase in the amount of ECM produced that encases the microcolonies into a structured microbial community (Fig.1.2 *C*, maturation phase) [31, 33]. In addition, it was recently shown that the dispersion of cells from the biofilm occurs through all the stages of the life cycle, although in higher proportion during the intermediate phase of growth [32]. Of note, not only biomedical devices can be colonized by *Candida* species, but also host surfaces, such as the oral cavity [34].

Overall, three major problems can be anticipated concerning *Candida* biofilms and its contribution to disease. First, the possibility of impairment of the medical device function, thus leading to its removal. Second, the biofilm eradication, since the cells within the biofilms display increased tolerance/resistance to antifungal agents in comparison with free-living, planktonic, cells. Third, the possibility of the development of disseminated disease and of the colonization of additional sites, from cells derived from biofilm dispersion (reviewed in [30, 33, 35]). Thus, the ability to adhere and ultimately form biofilms has been increasingly referred to as a potential *Candida* virulence factor [12, 14, 16, 36, 37].

Despite their recognized clinical relevance, research in *Candida* species biofilms is still in its infancy. It should be noted that although the majority of our understanding on biofilms has been gained from studies in *C. albicans*, this knowledge cannot be transposed to NCAC species biofilms or even to strains of the same species. In fact, some studies show that biofilm forming ability and biofilm features, such as morphology and ECM composition may be strain dependent [38, 39]. In addition, few studies have addressed the polimicrobial nature of biofilms, that are found in a clinical setting. For example, a clinical study showed the co-isolation of *Candida* species in eight patients, from a cohort of 54 individuals infected with human immunodeficiency virus [40]. Evidences from in vitro data, show that *C. albicans* has growth competitive advantages over *Candida dubliniensis*, being more evident in planktonic than in biofilm growing conditions [41]. An additional study has reported that *C. albicans* and other *Candida* species may interact in order to control biofilm growth, even though this is strictly dependent on the *Candida* species that dominates the biofilm [42]. The mechanisms that regulate the diversity and distribution of *Candida* species within biofilms are still not understood. However, similarly to what is observed for fungi-bacteria interactions [43] it cannot be discarded that extracellular molecules contribute, at least in part, to these inter-species interactions.

CANDIDA SPECIES EXTRACELLULAR MOLECULES

Together with the cell wall, secreted molecules may constitute an efficient means by which *Candida* cells can sense and interact with surrounding environment, and ultimately constitute virulence attributes. The set of protein secreted by *Candida* species is well documented, namely lipases, phospholipases and secreted aspartic proteinases (reviewed in [11-16, 44]). However, *Candida* cells may secrete into the extracellular milieu other molecules, including nutrients, metabolic by-products, and signaling molecules [45] that may contribute to fungal pathogenecity. In fact, in the last years, other extracellular molecules, besides proteins, are emerging in the literature as potential regulators of *Candida* biology. Table 1.1 aims to summarize *Candida* species extracellular molecules that have been reported to regulate fungal and/or host functions. However, it must be noted that some of these compounds may be found in literature with different names, due to the non-uniformity of the nomenclature used.

All the molecules described in Table 1.1 have been identified in *Candida* species culture planktonic supernatants. Only DNA [46] has been isolated from the biofilm ECM, prostaglandins [47], and tyrosol [48], have been identified in the supernatant of biofilm growing cells. In addition, the majority of the studies summarized in Table 1.1, focus on one single molecule. This limitation may result from the extraction and concentration methods applied and not from the analytical method, specifically gas chromatography-mass spectrometry, applied for example for the analysis of farnesol [49], and high performance liquid chromatography, used for the analysis of tyrosol [48].

Notably, a large group of these metabolites share an alcohol functional group, namely, *E*,*E*-farnesol (farnesol), isoamyl alcohol, *E*-nerolidol (nerolidol), 2-phenylethanol (phenylethanol), tryptophol, and tyrosol (Table 1.1). Although the biosynthetic pathways have not been characterized in *Candida* species, they have been assigned based on indirect evidences using inhibitors of specific pathways (e.g., [50]) or by analogy with biosynthetic pathways in *Saccharomyces cerevisiae* (e.g., [51]). Thus, based on their biosynthetic precursors they are classified as: aromatic (phenylethanol, tryptophol, and tyrosol) and aliphatic (isoamyl alcohol) alcohols, sesquiterpenoids (farnesol, farnesoic acid, and nerolidol), and lipids (prostaglandins and 3(*R*)-hydroxy-tetradecaenoic). Curiously, the main biosynthetic precursors of these molecules: amino acids, mevalonate, and fatty acids, together with shikimate and acetate are considered the "building blocks" of the secondary metabolites [52].

Table 1-1: Summary of molecules secreted by *Candida* species into the extracellular milieu

Extracellular molecule	*Candida* species	Biosynthetic precursor	(*Candida* species) Regulated function
DNA	*C. albicans*, *C. tropicalis*, *C. parapsilosis* [46, 53]	n.k.	(*C. albicans*), ‡Biofilm [54] ,host immunomodulator [55-57]
Farnesoic acid	*C. albicans* [58]	‡Mevalonate (farnesyl diphosphate) [59]	(*C. albicans*) Filamentation [58]
E,E-Farnesol	*C. albicans* [49], *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. kefyr*, *C. guilliermondii* [60]	‡Mevalonate (farnesyl diphosphate) [50, 61]	(*C. albicans*) Biofilm [62], chlamydospores formation [25], cross-kingdom communication (reviewed in [43, 63]), filamentation [49], †growth [64], host immunomodulator [65-68], mating [24], oxidative stress resistance [69] (*C. dubliniensis*) antifungal resistance, biofilm [70], filamentation, †growth [70, 71] (*C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. tropicalis*) †Biofilm [72-74], growth [72, 73]
3(*R*)-Hydroxy-tetradecaenoic acid	*C. albicans* [75]	‡Fatty acids (arachidonic acid) [75]	(*C. albicans*) Biofilm, filamentation [75]
Isoamyl alcohol	*C. krusei* [76]	‡Amino acids (leucine) [77]	(*C. albicans*) Filamentation [78]
E-Nerolidol	*C. krusei* [76]	‡Mevalonate (farnesyl diphosphate) [79]	(*C. albicans*) Filamentation [49]
2-Phenylethanol	*C. albicans* [80, 81], *C. krusei* [76]	‡Amino acids (phenylalanine) [51]	(*C. albicans*) Biofilm [81], †filamentation [81, 82], growth [80]
Prostaglandins	*C. albicans* [47, 83]	‡Fatty acids (arachidonic acid) [84]	(*C. albicans*) Filamentation [83]
Tryptophol	*C. albicans* [80, 81], *C. dubliniensis* [85]	‡Amino acids (tryptophan) [51]	(*C. albicans*) †Biofilm [81]; †filamentation [81, 82], growth [80]
Tyrosol	*C. albicans* [48, 86], *C. glabrata*, *C. parapsilosis*, *C. tropicalis* [87]	‡Amino acids (tyrosine) [51]	(*C. albicans*) †Biofilm [48, 81], filamentation, growth [86], protection against phagocytic killing [87]

n.k., not known; †, ambiguous effect, based on different reported information; ‡, presumptive pathway

Remarkably, the research on extracellular compounds has been centered in *C. albicans* (Table 1.1), with only few studies focusing on NCAC species [e.g. [73, 74, 76]]. Furthermore, the insights into *Candida* species extracellular molecules (Table 1.1) arose from studies evaluating one/two reference strains of each *Candida* species, with the use of mutant strains being restricted to *C. albicans* studies [46, 47, 51, 57, 67, 68, 88]. Of note, from these mutant strains only one defective in the production of the extracellular molecules has been generated, specifically the *C. albicans dpp3*Δ/*dpp3*Δ mutant strain, defective in one of the two genes that converts farnesyl pyrophosphate to farnesol [68].

Additionally, Table 1.1 shows the particular interest in the elucidation of the role of these molecules in the in vitro regulation of planktonic cells filamentation and growth, and biofilm development. In general, the strategy followed by the several researchers consisted on the evaluation of the effect of the exogenous addition of such compounds in order to infer the effect on the specific function evaluated. The ambiguous effect reported for the action of some compounds (e.g., tyrosol in *C. albicans* biofilm [48, 81]) may be related to the use of different strains and growth conditions, such as medium or compound concentration. Furthermore, it is observed an attempt to elucidate the immunomodulatory role of these molecules, specifically observed for farnesol and DNA (Table 1.1), evidencing the need to evaluate the in vivo effect of the compounds to establish a possible relationship with pathogenesis.

Finally, it is clear from the data reported in Table 1.1 that farnesol is by far the most extensively studied molecule (recently reviewed by Langford et al. [89]). This is probably due to the fact that it was the first quorum sensing molecule described in an eukaryote [49]. Quorum sensing was originally identified in bacteria as a mechanism of cells to respond to changes in cell population density. Cells produce, release, detect, and respond to threshold levels of autoinducers molecules in order to coordinate virulence factors expression [90]. As a quorum sensing molecule, farnesol accumulates in the extracellular medium during growth and regulates *C. albicans* filamentation upon a threshold level [49]. However, a farnesol specific receptor has not been identified yet, and this is a requirement that needs to be fulfilled for it to be considered a quorum sensing molecule [91]. In addition, farnesol has been shown to modulate the host immune system in a manner that is advantageous for *C. albicans*, being suggested to act as a virulence factor in this *Candida* species [65, 65, 67].

Overall, there is an increasing awareness that *Candida* species release other molecules than proteins, into the extracellular medium, that may regulate their species biology, and the ones presented in Table 1.1 may represent only the "tip of the iceberg". Thus, this work aimed to bring insights into *Candida* species world, following two main routes: first, the analysis of compounds released by *Candida* species into the extracellular medium focusing on extracellular DNA (Chapter 2) and alcohol compounds (Chapter 3), and to elucidate the interactions of the identified molecules with virulence traits of *Candida* species, and second, the identification of *Candida* species in a clinical context (Chapter 4).

REFERENCES

1. Pfaller, M. A. & Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**, 133-163.
2. Hazen, K. C. (1995) New and emerging yeast pathogens. *Clin Microbiol Rev* **8**, 462-478.
3. Lewis, R. E. (2009) Overview of the changing epidemiology of candidemia. *Curr Med Res Opin* **25**, 1732-1740.
4. Loreto, E. S., Scheid, L. A., Nogueira, C. W., Zeni, G., Santurio, J. M., & Alves, S. H. (2010) *Candida dubliniensis*: epidemiology and phenotypic methods for identification. *Mycopathologia* **169**, 431-443.
5. Hennen, C. R. (2009) Pharmacoeconomic evaluations of antifungal therapies. *Curr Med Res Opin* **25**, 1751-1758.
6. Odds, F. C. *Candida* research: past, present and future. Communication at *10th Candida and Candidiasis Conference*, Miami March 22nd - 26th. 2010.
7. Soll, D. R., Galask, R., Schmid, J., Hanna, C., Mac, K., & Morrow, B. (1991) Genetic dissimilarity of commensal strains of *Candida* spp. carried in different anatomical locations of the same healthy women. *J Clin Microbiol* **29**, 1702-1710.
8. Uppuluri, P. Gene expression studies in *Candida albicans* (*PhD dissertation*). 2006. Graduate Faculty of Texas Tech University Health Sciences Center.
9. Kullberg, B. J. & Filler, S. G. (2002) Candidemia In: Calderone, R. A. (ed) *Candida and Candidiasis*. Washington: ASM, pp 327-340.
10. Gullo, A. (2009) Invasive fungal infections: the challenge continues. *Drugs* **69**, 65-73.
11. Calderone, R. A. & Fonzi, W. A. (2001) Virulence factors of *Candida albicans*. *Trends Microbiol* **9**, 327-335.
12. Li, L., Redding, S., & Dongari-Bagtzoglou, A. (2007) *Candida glabrata*: an emerging oral opportunistic pathogen. *J Dent Res* **86**, 204-215.
13. Haynes, K. (2001) Virulence in *Candida* species. *Trends Microbiol* **9**, 591-596.
14. Trofa, D., Gacser, A., & Nosanchuk, J. D. (2008) *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**, 606-625.

15. Sullivan, D. J., Moran, G. P., & Coleman, D. C. (2005) *Candida dubliniensis*: Ten years on. *FEMS Microbiol Lett* **253**, 9-17.
16. Samaranayake, Y. H. & Samaranayake, L. P. (1994) *Candida krusei*: biology, epidemiology, pathogenicity and clinical manifestations of an emerging pathogen. *J Med Microbiol* **41**, 295-310.
17. Falkow, S. (1988) Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis* **10**, S274-S276.
18. Carlisle, P. L., Banerjee, M., Lazzell, A., Monteagudo, C., Lopez-Ribot, J. L., & Kadosh, D. (2009) Expression levels of a filament-specific transcriptional regulator are sufficient to determine *Candida albicans* morphology and virulence. *Proc Natl Acad Sci USA* **106**, 599-604.
19. Calderone, R. A. (2002) Taxonomy and biology of *Candida*. In: Calderone, R. A. (ed) *Candida and Candidiasis*. Washington: ASM, pp 15-27.
20. Stokes, C., Moran, G. P., Spiering, M. J., Cole, G. T., Coleman, D. C., & Sullivan, D. J. (2007) Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet Biol* **44**, 920-931.
21. Ernst, J. F. (2000) Transcription factors in *Candida albicans* - environmental control of morphogenesis. *Microbiology* **146**, 1763-1774.
22. Biswas, S., Van, D. P., & Datta, A. (2007) Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* **71**, 348-376.
23. Whiteway, M. & Bachewich, C. (2007) Morphogenesis in *Candida albicans*. *Annu Rev Microbiol* **61**, 529-553.
24. Dumitru, R., Navarathna, D. H., Semighini, C. P. *et al.* (2007) In vivo and in vitro anaerobic mating in *Candida albicans*. *Eukaryot Cell* **6**, 465-472.
25. Martin, S. W., Douglas, L. M., & Konopka, J. B. (2005) Cell cycle dynamics and quorum sensing in *Candida albicans* chlamydospores are distinct from budding and hyphal growth. *Eukaryot Cell* **4**, 1191-1202.
26. Ramage, G., Saville, S. P., Thomas, D. P., & Lopez-Ribot, J. L. (2005) *Candida* biofilms: an update. *Eukaryot Cell* **4**, 633-638.
27. Bastidas, R. J. & Heitman, J. (2009) Trimorphic stepping stones pave the way to fungal virulence. *Proc Natl Acad Sci USA*. **106**, 351-352.
28. Kumamoto, C. A. & Vinces, M. D. (2005) Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell Microbiol* **7**, 1546-1554.
29. Collin, B., Clancy, C. J., & Nguyen, M. H. (1999) Antifungal resistance in Non-*albicans Candida* species. *Drug Resist Updat* **2**, 9-14.
30. Ramage, G., Martinez, J. P., & Lopez-Ribot, J. L. (2006) *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* **6**, 979-986.
31. Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., & Ghannoum, M. A. (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**, 5385-5394.
32. Uppuluri, P., Chaturvedi, A. K., Srinivasan, A. *et al.* (2010) Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog* **6**, e1000828.

33. Ramage, G., Mowat, E., Jones, B., Williams, C., & Lopez-Ribot, J. (2009) Our current understanding of fungal biofilms. *Crit Rev Microbiol* **35**, 340-355.
34. Cannon, R. D., Holmes, A. R., Mason, A. B., & Monk, B. C. (1995) Oral *Candida*: clearance, colonization, or candidiasis? *J Dent Res* **74**, 1152-1161.
35. Blankenship, J. R. & Mitchell, A. P. (2006) How to build a biofilm: a fungal perspective. *Curr Opin Microbiol* **9**, 588-594.
36. Thein, Z. M., Seneviratne, C. J., Samaranayake, Y. H., & Samaranayake, L. P. (2009) Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* **52**, 467-475.
37. Negri, M., Martins, M., Henriques, M., Svidzinski, T. I., Azeredo, J., & Oliveira, R. (2010) Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients. *Mycopathologia* **169**, 175-182.
38. Silva, S., Henriques, M., Martins, A., Oliveira, R., Williams, D., & Azeredo, J. (2009) Biofilms of Non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* **47**, 681-689.
39. Henriques, M., Azeredo, J., & Oliveira, R. (2006) *Candida albicans* and *Candida dubliniensis*: comparison of biofilm formation in terms of biomass and activity. *Br J Biomed Sci* **63**, 5-11.
40. Jabra-Rizk, M. A., Ferreira, S. M., Sabet, M., Falkler, W. A., Merz, W. G., & Meiller, T. F. (2001) Recovery of *Candida dubliniensis* and other yeasts from human immunodeficiency virus-associated periodontal lesions. *J Clin Microbiol* **39**, 4520-4522.
41. Kirkpatrick, W. R., Lopez-Ribot, J. L., Mcatee, R. K., & Patterson, T. F. (2000) Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *J Clin Microbiol* **38**, 902-904.
42. El-Azizi, M. A., Starks, S. E., & Khardori, N. (2004) Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *J Appl Microbiol* **96**, 1067-1073.
43. Shirtliff, M. E., Peters, B. M., & Jabra-Rizk, M. A. (2009) Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett* **299**, 1-8.
44. Ramage, G., Tomsett, K., Wickes, B. L., Lopez-Ribot, J. L., & Redding, S. W. (2004) Denture stomatitis: a role for *Candida* biofilms. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **98**, 53-59.
45. Williams, P., Winzer, K., Chan, W. C., & Camara, M. (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R. Soc Lond B Biol Sci* **362**, 1119-1134.
46. Paramonova, E., Krom, B. P., van der Mei, H. C., Busscher, H. J., & Sharma, P. K. (2009) Hyphal content determines the compression strength of *Candida albicans* biofilms. *Microbiology* **155**, 1997-2003.
47. Alem, M. A. S. & Douglas, L. J. (2005) Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* **54**, 1001-1005.
48. Alem, M. A., Oteef, M. D., Flowers, T. H., & Douglas, L. J. (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell* **5**, 1770-1779.
49. Hornby, J. M., Jensen, E. C., Lisec, A. D. *et al.* (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982-2992.

50. Hornby, J. M., Kebaara, B. W., & Nickerson, K. W. (2003) Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. *Antimicrob Agents Chemother* **47**, 2366-2369.
51. Ghosh, S., Kebaara, B. W., Atkin, A. L., & Nickerson, K. W. (2008) Regulation of aromatic alcohol production in *Candida albicans*. *Appl Environ Microbiol* **74**, 7211-7218.
52. Mann, J. (1999) *Chemical aspects of biosynthesis*. 2nd ed.New York: Oxford University Press Inc.
53. Kasai, M., Francesconi, A., Petraitiene, R. *et al.* (2006) Use of quantitative real-time PCR to study the kinetics of extracellular DNA released from *Candida albicans*, with implications for diagnosis of invasive candidiasis. *J Clin Microbiol* **44**, 143-150.
54. Al-Fattani, M. A. & Douglas, L. J. (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**, 999-1008.
55. Dimitrova, P., Yordanov, M., Danova, S., & Ivanovska, N. (2008) Enhanced resistance against systemic *Candida albicans* infection in mice treated with *C. albicans* DNA. *FEMS Immunol Med Microbiol* **53**, 231-236.
56. Yordanov, M., Dimitrova, P., Danova, S., & Ivanovska, N. (2005) *Candida albicans* double-stranded DNA can participate in the host defense against disseminated candidiasis. *Microbes Infect* **7**, 178-186.
57. Miyazato, A., Nakamura, K., Yamamoto, N. *et al.* (2009) Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*. *Infect Immun* **77**, 3056-3064.
58. Oh, K. B., Miyazawa, H., Naito, T., & Matsuoka, H. (2001) Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc Natl Acad Sci USA* **98**, 4664-4668.
59. Kim, S., Kim, E., Shin, D. S., Kang, H., & Oh, K. B. (2002) Evaluation of morphogenic regulatory activity of farnesoic acid and its derivatives against *Candida albicans* dimorphism. *Bioorg Med Chem Lett* **12**, 895-898.
60. Weber, K., Sohr, R., Schulz, B., Fleischhacker, M., & Ruhnke, M. (2008) Secretion of *E,E*-farnesol and biofilm formation in eight different *Candida* species. *Antimicrob Agents Chemother* **52**, 1859-1861.
61. Nickerson, K. W., Atkin, A. L., & Hornby, J. M. (2006) Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl Environ Microbiol* **72**, 3805-3813.
62. Ramage, G., Saville, S. P., Wickes, B. L., & Lopez-Ribot, J. L. (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* **68**, 5459-5463.
63. DeSordi, L. & Muhlschlegel, F. A. (2009) Quorum sensing and fungal-bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence. *FEMS Yeast Res.* **9**, 990-999.
64. Langford, M. L., Hasim, S., Nickerson, K. W., & Atkin, A. L. (2010) Activity and toxicity of farnesol towards *Candida albicans* is dependent on growth conditions. *Antimicrob Agents Chemother* **54**, 940-942.
65. Navarathna, D. H., Nickerson, K. W., Duhamel, G. E., Jerrels, T. R., & Petro, T. M. (2007) Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect Immun* **75**, 4006-4011.
66. Hisajima, T., Maruyama, N., Tanabe, Y. *et al.* (2008) Protective effects of farnesol against oral candidiasis in mice. *Microbiol Immunol* **52**, 327-333.

67. Ghosh, S., Howe, N., Volk, K., Tati, S., Nickerson, K. W., & Petro, T. M. (2010) *Candida albicans* cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. *FEMS Immunol Med Microbiol* **in press**.
68. Navarathna, D. H., Hornby, J. M., Krishnan, N., Parkhurst, A., Duhamel, G. E., & Nickerson, K. W. (2007) Effect of farnesol on a mouse model of systemic candidiasis: a DPP3 knock out mutant of *Candida albicans*. *Infect Immun* **75**, 1609-1618.
69. Westwater, C., Balish, E., & Schofield, D. A. (2005) *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. *Eukaryot Cell* **4**, 1654-1661.
70. Jabra-Rizk, M. A., Shirtliff, M., James, C., & Meiller, T. (2006) Effect of farnesol on *Candida dubliniensis* biofilm formation and fluconazole resistance. *FEMS Yeast Res* **6**, 1063-1073.
71. Henriques, M., Martins, M., Azeredo, J., & Oliveira, R. (2007) Effect of farnesol on *Candida dubliniensis* morphogenesis. *Lett Appl Microbiol* **44**, 199-205.
72. Weber, K., Schulz, B., & Ruhnke, M. (2010) The quorum-sensing molecule *E,E*-farnesol-its variable secretion and its impact on the growth and metabolism of *Candida* species. *Yeast* **in press**.
73. Rossignol, T., Logue, M. E., Reynolds, K., Grenon, M., Lowndes, N. F., & Butler, G. (2007) Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob Agents Chemother* **51**, 2304-2312.
74. Laffey, S. F. & Butler, G. (2005) Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151**, 1073-1081.
75. Nigam, S., Ciccoli, R., Ivanov, I., Sczepanski, M., & Deva, R. (2010) On mechanism of quorum sensing in *Candida albicans* by 3(R)-hydroxy-tetradecaenoic acid. *Curr Microbiol* **in press**.
76. Annan, N. T., Poll, L., Sefa-Dedeh, S., Plahar, W. A., & Jakobsen, M. (2003) Volatile compounds produced by *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* in single starter culture fermentations of Ghanaian maize dough. *J Appl Microbiol* **94**, 462-474.
77. Dickinson, J. R., Lanterman, M. M., Danner, D. J. *et al.* (1997) A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 26871-26878.
78. Dickinson, J. R. (1996) 'Fusel' alcohols induce hyphal-like extensions and pseudohyphal formation in yeasts. *Microbiology* **142**, 1391-1397.
79. Zea, L., Moreno, J., Ortega, J. M., & Medina, M. (1995) Content of free terpenic compounds in cells and musts during vinification with three *Saccharomyces cerevisiae* races. *J Agric Food Chem* **43**, 1110-1114.
80. Lingappa, B. T., Prasad, M., Lingappa, Y., Hunt, D. F., & Biemann, K. (1969) Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans*. *Science* **163**, 192-194.
81. Chen, H. & Fink, G. R. (2006) Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev* **20**, 1150-1161.
82. Hazen, K. C. & Cutler, J. E. (1979) Autoregulation of germ tube formation by *Candida albicans*. *Infect Immun* **24**, 661-666.

83. Noverr, M. C., Phare, S. M., Toews, G. B., Coffey, M. J., & Huffnagle, G. B. (2001) Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* **69**, 2957-2963.
84. Rhome, R. & Del, P. M. (2009) Lipid signaling in pathogenic fungi. *Annu Rev Microbiol* **63**, 119-131.
85. Kosalec, I., Puel, O., Delaforge, M. *et al.* (2008) Isolation and cytotoxicity of low-molecular-weight metabolites of *Candida albicans*. *Front Biosci.* **13**, 6893-6904.
86. Chen, H., Fujita, M., Feng, Q., Clardy, J., & Fink, G. R. (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc Natl Acad Sci USA* **101**, 5048-5052.
87. Cremer, J., Vatou, V., & Braveny, I. (1999) 2,4-(hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing in vitro. *FEMS Microbiol Lett* **170**, 319-325.
88. Kebaara, B. W., Langford, M. L., Navarathna, D. H., Dumitru, R., Nickerson, K. W., & Atkin, A. L. (2008) *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous growth induction. *Eukaryot Cell* **7**, 980-987.
89. Langford, M. L., Atkin, A. L., & Nickerson, K. W. (2009) Cellular interactions of farnesol, a quorum-sensing molecule produced by *Candida albicans*. *Future. Microbiol.* **4**, 1353-1362.
90. Parsek, M. R. & Greenberg, E. P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13**, 27-33.
91. Winzer, K., Hardie, K. R., & Williams, P. (2002) Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **5**, 216-222.

CHAPTER 2 Insights into *Candida* world: extracellular DNA

Chapter 2.1 Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms

Abstract

DNA has been described as a structural component of the extracellular matrix (ECM) in bacterial biofilms. In *Candida albicans* there is a scarce knowledge concerning the contribution of extracellular DNA (eDNA) to biofilm ECM and overall structure. This work examined the presence and quantified the amount of eDNA in *C. albicans* biofilm ECM and the effect of deoxyribonuclease I (DNase) treatment and the addition of exogenous DNA on *C. albicans* biofilm development as indicators of a role for eDNA in biofilm development. We were able to detect the accumulation of eDNA in biofilm ECM extracted from *C. albicans* biofilms formed under conditions of flow, although the quantity of eDNA detected differed according to growth conditions, in particular with regards to the medium used to grow the biofilms. Experiments with *C. albicans* biofilms formed statically using a microtiter plate model indicated that DNase treatment (> 0.03 mg/ml) decreases biofilm biomas, and, conversely addition of exogenous DNA (>160 ng/ml) increases biofilm biomass, at later time points of biofilm development. We present evidence for the role of eDNA in *C. albicans* biofilm structure and formation, consistent with eDNA being a key element of the ECM in mature *C. albicans* biofilms and playing a predominant role in biofilm structural integrity and maintenance.

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INTRODUCTION

Biofilms are structurally complex microconsortia of surface adhering cells embedded within a extracellular matrix (ECM) composed of substances produced and secreted by cells or derived from cell lysis [1]. The ECM contributes to the architectural preservation of biofilms by the maintenance of stable cell–cell and cell–surface interactions, and acts as a protective barrier [2]. Although polysaccharides and proteins are the more extensively studied substances of biofilms ECM, other molecules such as lipids and nucleic acids play a crucial role on ECM functions [1]. In fact, extracellular DNA (eDNA) has been identified in the surrounding milieu as part of the ECM of biofilms formed by both Gram-negative species such as *Pseudomonas aeruginosa* [3] and Gram-positive bacteria such as *Bacillus cereus* [4]. The proposed mechanisms implicated in eDNA release include: (i) cell lysis, (ii) quorum sensing, and (iii) excretion from DNA containing vesicles [3]. Bacterial biofilms eDNA characterization revealed that it comprises fragments of high molecular weight (around 30 Kb) [5] that differ from genomic DNA, as indicated by the different profiles exhibited upon restriction endonuclease treatments or randomly amplified polymorphic DNA analysis [6, 7]. Evidence for a role of eDNA as a structural component of bacterial biofilms ECM arose from studies showing that eDNA is required at the initial stages of biofilm formation [8, 9]. Additionally, independent studies evaluating exogenous DNA and DNase effect on biofilm cells antimicrobial susceptibility [5, 10] showed an association between eDNA and biofilms increased antibiotic resistance.

Despite the intensive research in the bacterial field, the investigation focusing on eDNA in fungal biofilms is scarce.

C. albicans is a polymorphic fungus that causes opportunistic infections in humans and its ability to form biofilms is well characterized [11]. *C. albicans* ECM abundance was recently shown to be regulated positively by the glucoamylases Gca1 and Gca2 and by the alcohol dehydrogenase Adh5, and negatively by the alcohol dehydrogenases Csh1 and Ifd6 [12]. *C. albicans* ECM consists predominantly of carbohydrates but also contains proteins, hexosamine, uronic acid, and phosphorus [13]. Consistent with the presence of phosporous, circumstantial evidences points to the presence of eDNA in ECM. Specifically, mature biofilms treatment with 50 µg/ml of deoxyribonuclease I (DNase) resulted in 30% decrease of biofilm biomass, similarly to the observed for proteinase K treatments [13]. More recently, eDNA was extracted from *C.*

albicans 72 h biofilm ECM within micrograms per gram of biofilm wet weight [14]. Additionally, it has been demonstrated that the presence of eDNA is a common feature of biofilms formed by other *Candida* species [14].

However, further studies are required to extend the knowledge on the contribution of eDNA to *C. albicans* biofilms structure and matrix composition. Here we examined the presence of DNA in *C. albicans* biofilm ECM and the effect of DNase and the addition of exogenous *C. albicans* DNA on biofilm formation.

Material and Methods

Strain and culture conditions

The *C. albicans* wild type strain SC5314 was used in this study. Cells were stored at -70°C in 20% glycerol stocks and propagated by streaking a loopfull of cells onto Sabouraud dextrose agar medium (BD, Franklin Lakes, NJ, USA) supplemented with 100 mg/l ampicillin (Fisher Bioreagents, Fair Lawn, NJ, USA) and incubating at 30°C for 24 h. These stocks were stored at 4°C for no longer than two weeks. For all experiments, batches (30 ml in 125 ml flasks) of yeast extract-peptone-dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] (US Biological, Swampscott, MA) were inoculated with freshly grown yeast cells in an orbital shaker at 30°C for 24 h. Cells were harvested by centrifugation and washed in sterile saline. Cells were counted with a haemocytometer and dilutions were made to prepare standardized cell suspensions (1×10^6 cells/ml) in the appropriate pre-warmed growth medium (see below). Confirmation of the number and viability of cells was determined by plating serial dilutions on Sabouraud dextrose agar. Colony forming units (CFU) were counted after 24-h incubation at 30°C.

eDNA estimation in *C. albicans* biofilm ECM

Biofilm formation under conditions of flow

Three different influent media were used (i) Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with L-glutamine (Mediatech Inc., Herdon, VA, USA), sodium

bicarbonate [0.2% (w/v)], and buffered with 0.165 M 3-[N-morpholino] propanesulfonic acid) (Research Products International Corp., Mount Prospect, IL, USA), final pH 7, (ii) YPD and (iii) yeast nitrogen base (YNB) (Fluka, St. Louis, MO, USA) supplemented with glucose [0.9% (w/v)] at final pH 7. The culture media used in the flow system were diluted in order to promote biofilm formation. Specifically, the chemically defined media RPMI and YNB were diluted 1:1 whereas the rich medium YPD was diluted 1:10. The media were supplemented with ampicillin 100 mg/l. Culturing conditions and apparatus were established as previously described [15]. Briefly, silicone elastomer (SE) strips (1 × 9 cm^2) (Cardiovascular Instrument Corp. Silicone sheets, Wakefield, MA) were washed twice with ultrapure water, sterilized by autoclaving and pretreated overnight at 30°C with foetal calf serum (Sigma, St. Louis, MO, USA). *C. albicans* standardized cell suspensions (13 ml) were prepared using the same growth medium as the influent (1 × concentrated) and incubated with the SE at 37°C, 130 rpm for 90 min. After the adhesion period, the SE were transferred to the flow system and influent medium was set up at an initial flow rate of 0.75 ml/min [15]. Biofilms were formed at 37°C for 48 h. For subsequent analysis, two SE with biofilm were pooled together. Two independent experiments were performed for each growth medium condition.

Planktonic cultures (50 ml of standardized cell suspension per 125 ml flask; 1 × concentrated YPD, RPMI or YNB) were grown at 37°C and 130 rpm for 48 h to validate the isolation of biofilm ECM (see below).

Isolation of biofilm ECM

ECM was isolated as previously described [16] with slight modifications. Briefly, after formation the biofilms were transferred to polypropylene conical tubes (BD), resuspended in 10 ml of ultrapure sterile water and vortexed for 1 min. Next, biofilm cells were sonicated in an ultrasonic bath for 45 min, followed by a vortexing step of 2 min and centrifuged (2000 rpm; 20 min). The supernatant fraction was recovered and filtered through 0.2 μm acrodisc syringe filters with Supor Membrane (Pall Life Sciences, Ann Arbor, MI, USA) that are low protein and nucleic acids binding membranes. Planktonic cultures were processed in parallel with biofilms during ECM extraction and aliquots were taken before and after the sonication step. The confirmation of planktonic cultures viability was performed by CFU counts in Sabouraud dextrose agar medium.

ECM analyses

The DNA of the ECM fraction was extracted with an equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1, v/v) (Invitrogen, Carlsbad, CA, USA) and then precipitated with isopropanol (1:1; v/v). After centrifugation, the pellet was rinsed with 70% ethanol and air dried. The pellet was dissolved in 20 µl of Tris–EDTA (TE) buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA].

DNA was quantified using the Quant-iT^{TM} ds BR Assay kit (Molecular Probes, Invitrogen) according to the suppliers' instructions. This assay allows an accurate determination of DNA between 100 ng/ml and 1000 ng/ml. A λ DNA standard 45.7 µg/ml was prepared in TE buffer from a commercially available standard solution (Promega, Madison, WI, USA) and run in parallel with samples in each assay. Additionally, a blank (TE only) was included in each assay. Samples were read in the Quibit^{TM} fluorometer (Molecular Probes, Invitrogen) and a new calibration was run for each assay. A minimum of one DNA measurement was performed for biofilm samples.

The protein content of the ECM fraction was estimated using the EZQ® Protein Quantification kit (Molecular Probes, Invitrogen). Standards for the assay were prepared in nanopure water (Biorad, Hercules, CA, USA) with ovalbumin supplied with the kit. Standard concentrations range was between 0 and 0.25 mg/ml in order to include sample concentrations. Fotodyne UV 300 transiluminator (Fisher) was used for UV measurements. Standard curves were generated for mean UV intensity vs ovalbumin concentration. All measurements were made with three replicates.

Effect of DNase and exogenous DNA treatment on *C. albicans* biofilm formation

Deoxyribonuclease I (DNase) from bovine pancreas (Sigma) stock solutions were prepared before each experiment in saline supplemented with 5 mM of $MgCl_2$ (Merck, VWR, West Chester, PA, USA). The range of concentrations chosen was based on previously described titration data for bacterial species [17]. Working solutions of DNase in two-fold increments ranging from 0.02 to 2 mg/ml were prepared in RPMI medium.

C. albicans genomic DNA, to be added as exogenous DNA for *C. albicans* cells treatment, was extracted with the MasterPure™ Yeast DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the suppliers' instructions. *C. albicans* DNA purity and concentration were estimated by measuring the A_{260} and A_{280} (Eppendorf Biophotometer, Eppendorf, USA). It has been previously described that in *C. albicans* stationary phase planktonic cultures eDNA levels are higher than 0.1 ng/ml [18]. In this sense, physiological and supraphysiological concentrations ranging from 10 to 2,560 to ng/ml, with two-fold increments, were chosen for this assay. Dilutions of the genomic DNA stock solution were prepared in RPMI medium.

For control experiments, DNase was heat inactivated at 95°C for 10 min, and Ribonuclease (RNase) (Sigma) stock solutions were prepared in TE buffer.

DNase treatment and exogenous DNA addition at different stages of biofilm formation

Standardized cell suspensions (100 µl) were inoculated into polystyrene, flat-bottomed, 96-well microtitre plates (Corning Inc., Corning, NY, USA) and incubated for 0, 1, 2 and 24 h at 37°C under static conditions. At 0-h (preincubation), DNase was added to the standardized suspension. At 1-, 2- and 24-h of incubation the medium was removed and adhered cells washed two times with sterile phosphate buffered saline (PBS) (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma). DNase working solutions were then added to *C. albicans* cells. The plates were incubated at 37°C for additional 24 h. The same procedure was performed for the addition of exogenous DNA to *C. albicans* cells. Positive controls for biofilm formation included RPMI only and RPMI plus 5 mM of $MgCl_2$ for DNase experiments. Negative background control for subsequent analysis consisted of RPMI medium only. Four independent assays with three to eight replicates for each condition were performed. The effect of DNase treatment and exogenous DNA treatments was examined in terms of biofilm biomass changes using the crystal violet assay (see below).

As controls for DNase enzymatic activity, RNase and heat inactivated DNase were added at 24 h as described above. This experiment was performed once with eight replicates.

The effect of DNase and/ or exogenous DNA on *C. albicans* viability and filamentation was examined in planktonic cultures and on preformed biofilms. For planktonic cultures, 5 ml of the standardized cell suspension plus (i) 5 mM of $MgCl_2$, (ii) 2 mg/ml DNase, and (iii) 2,560 ng/ml of exogenous DNA were incubated at 37°C, 130 rpm for 2 h. Viability was examined by CFU counts in Sabouraud dextrose agar medium. Filamentation was monitored by light microscopy using an inverted microscope (Westover Scientific, Mill Creek, WA, USA). These experiments were performed twice. The highest concentrations of DNase and exogenous DNA used in these experiments did not have an effect on *C. albicans* planktonic cells viability and filamentation. For preformed biofilms, DNase ranging from 2 to 0.02 mg/ml was added to 24-h mature biofilms as described above and incubated for an additional 24 h. Next, cells were removed with a micropipette tip, re-suspended in saline solution and CFU analysis carried out. Light microscopy examination of biofilms was performed in parallel. All the treatments were compared with the untreated (RPMI and RPMI plus 5 mM $MgCl_2$) controls.

Examination of the effect of DNase treatment and exogenous DNA on biofilm formation

The effect of DNase and exogenous DNA on *C. albicans* biofilm biomass was estimated using the crystal violet assay. Briefly, the medium was removed and biofilms washed twice with PBS. Then, biofilms were stained with 50 µl of crystal violet 3 g/l [0.3% (w/v) crystal violet (Sigma), 5% (v/v) isopropanol, 5% (v/v) methanol, 90% (v/v) water] for 5 min. Afterwards, each well was washed twice with PBS, air dried and destained with 100 µl of ethanol 100%. Next, 75 µl of the destaining solution was transferred to a new microtiter plate, and the A_{550} was measured (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA). Samples exhibiting very intense color, yielding "offscale" absorbance values, were diluted before performing a second absorbance reading.

Statistical analyses

The association between the *C. albicans* 48-h biofilm ECM eDNA/protein content in the different experimental conditions was analysed using the chi-square test [19]. A statistical confidence interval of 95% was established. The comparison between (i) log CFU/ml before and after

sonication and (ii) each biofilm treatment and control condition was performed by two-tailed unpaired t-test (confidence interval 95%) using GraphPad Prism, version 5.00 software for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P< 0.05$.

RESULTS

Presence of eDNA on *C. albicans* biofilm ECM

Three different media were tested for their ability to promote the accumulation of eDNA in the ECM of 48-h *C. albicans* biofilms developed under flow conditions.

In the extraction of ECM it was mandatory to ensure that there was no leak of cellular DNA during sample processing. To validate the ECM extraction process 48 h planktonic cultures were handled in parallel with biofilm samples. Before and after samples processing, aliquots were collected and CFU analysis carried out. Difference between means log CFU/ml before and after sonication were of: 0.00 (P= 1.00) for RPMI, -0.07 (P= 0.39) for YPD, and 0.00 (P= 1.00) for YNB.

As shown in Table 2.1-1, irrespective of which medium was used, eDNA was detected at measurable levels in ECM of *C. albicans* biofilms. Under the conditions used in this assay, the highest levels of eDNA in ECM were observed for biofilms formed using RPMI medium (3,045.4 ± 227.3 ng eDNA/mg of protein). These levels were 9-fold higher than those observed in the

Table 2.1-1. *C. albicans* 48-h biofilm ECM eDNA and protein content. DNA was extracted and further quantified. Protein content was directly assayed from the samples. Data presented represents mean ± mean deviation

Influent Medium	eDNA[a] (ng)	Protein[a] (mg)	eDNA/ protein (ng/mg)
RPMI[b]	1518 ± 822	0.48 ± 0.2	3m045.4 ± 227.3
YPD[c]	180 ± 160	0.42 ± 0.1	339.6 ± 265.6
YNB[b]	1.8 ± 1.6	0.10 ±0.1	13.0 ± 8.8

[a] obtained from biofilms developed under flow conditions, corresponding to 18 cm^2 of area; [b] used at 1:1 dilution; [c] used at YPD 1:10 dilution

Table 2.1-2. Results of the statistical analysis (P values) of *C. albicans* 48-h biofilm ECM quantification (ng eDNA/ mg of protein) obtained from the different experimental conditions

Influent Media	P value
RPMI vs YPD	< 0.01
YPD vs YNB	< 0.01
YNB vs RPMI	< 0.01

ECM of biofilms grown in YPD (Table 2.1-1; P< 0.01, Table 2.1-2). Both the absolute quantities of eDNA and the ratio of eDNA/protein were lowest in the ECM from YNB-grown biofilms when compared to biofilms grown in RPMI and YPD (Tables 2.1-1 and 2.1-2).

This data shows that eDNA is a component of *C. albicans* biofilm matrix and suggests that its accumulation in the ECM is affected by culture conditions.

Effect of DNase treatment on *C. albicans* biofilm formation

In order to examine the possible role of eDNA on *C. albicans* biofilm development, in a first set of experiments DNase was added to cells at different times (0, 1, 2 and 24 h) in the wells of microtiter plates and biofilm formation allowed to proceed for an additional 24 h (Fig. 2.1-1). The results of these experiments, assessed by a colorimetric assay for biofilm biomass showed that DNase did not exhibit major effects on *C. albicans* adherent cells at early time points during biofilm development (Fig.2.1-1). DNase was also used to treat preformed biofilms (24 h) to determine whether different concentrations of this enzyme could affect the formed biofilms. As shown in Fig.2.1-1, DNase showed a general inhibitory effect on *C. albicans* preformed biofilms. This was reflected by lower crystal violet readings at DNase concentrations higher than 0.03 mg/ml. For example, biofilms treated for 24 h with 0.13 mg/ml DNase showed a reduction of 40% in A_{550} readings when compared with the control (P= 0.03). Furthermore, preformed biofilms treatment with RNase and heat inactivated DNase at the same concentrations used for DNase did not display a decrease in biofilm biomass (data not shown), demonstrating that DNase activity is required for the observed effect.

Additionally, it should be noted that the microscopic examination of biofilms before washing suggested that the DNase treated biofilms (Fig.2.1-2 *I-b*) resembled the control

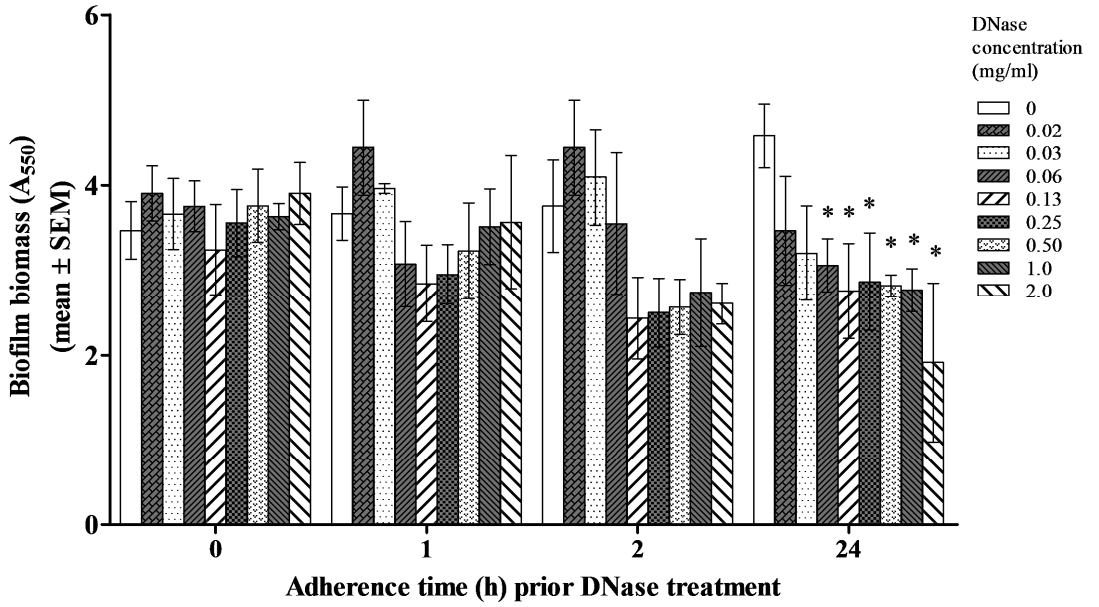

Fig.2.1-1. Effect of DNase treatment on *C. albicans* biofilm formation. Different DNase concentrations (0, 0.02, 0.03, 0.06, 0.13, 0.25, 0.50, 1.00 and 2.00 mg/ml) were added to *C. albicans* cells at different times (0, 1, 2 and 24 h) of incubation in the wells of microtiter plates and incubated at 37°C under static conditions. The extent of biofilm formation was estimated by the crystal violet assay. Presented values are mean A_{550} ± standard error of mean (SEM) of four independent experiments with three to eight replicates. Statistically significant differences (compared to biofilms formed in the absence of DNase) are indicated with an asterisk. (*, $P < 0.05$).

biofilms (Fig.2.1-2 *I-a*). However, during the biofilm processing for the determination of biofilm biomass, the DNase treated biofilms did detach from the microtiter plate surface (Fig.2.1-2 *II-d*), resulting in decreased A_{550}, in contrast to non-treated biofilms (Fig.2.1-2 *II-c*). Accordingly, the assessment of cell viability of pre-washed biofilms did not reveal a detrimental effect of DNase on biofilm cells viability (compared with the control). For example, for 24 h treatment with 0.13 mg/ml DNase the log CFU/well was 6.1 vs 6.5 for control biofilms.

This suggests that the observed effect induced by DNase treatment is not due to changes in biofilm cells growth or filamentation, but rather due to a direct effect on the biofilm ECM which is important for biofilm integrity and maintenance.

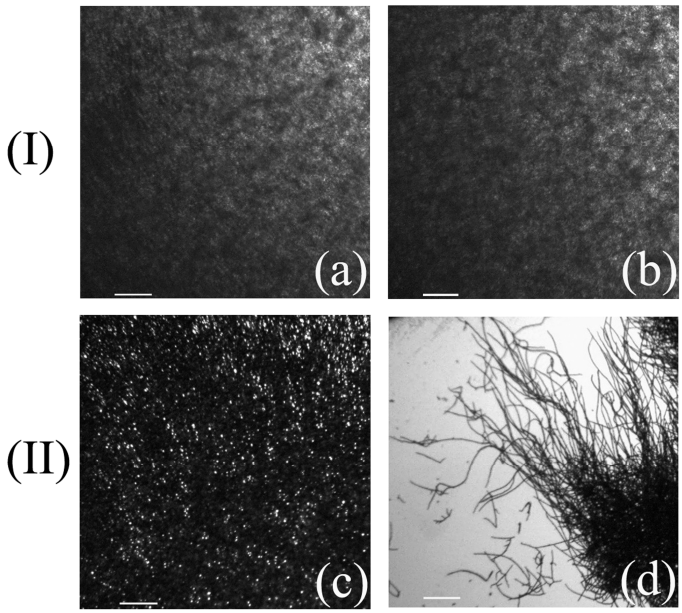

Fig.2.1-2. Example of microscopy evaluation of the effect of DNase treatment on 24 h *C. albicans* biofilm formation. Microphotographs of cells in wells before (***I***) and after (***II***) aspiration of medium and subsequent washings with PBS for biofilms treated with 0 mg/ml (***a*** and ***c***) and 0.13 mg/ml of DNase (***b*** and ***d***). The bar in the picture represents 200 μm.

Effect of addition exogenous DNA on *C. albicans* biofilm formation

To further substantiate the effect of eDNA as a structural component of biofilms, exogenous DNA was added to *C. albicans* cells. As shown in Fig.2.1-3, the addition of exogenous DNA to *C. albicans* adhered cell populations did not affect further biofilm development. In contrast, addition of exogenous DNA, at concentrations higher than 160 ng/ml to mature biofilms led to increases in biofilm biomass , as indicated by the differences in A_{550} readings compared with control biofilms (grown in the absence of exogenous DNA) (Fig.2.1-3). As an example, under the exposure to 320 ng/ml of exogenous DNA, biofilm biomass increased up to 63% in comparison with untreated biofilms (P= 0.03).

These experiments indicate that eDNA may contribute to the maintenance and stability of *C. albicans* mature biofilms, but is not required for the early stages of biofilms establishment.

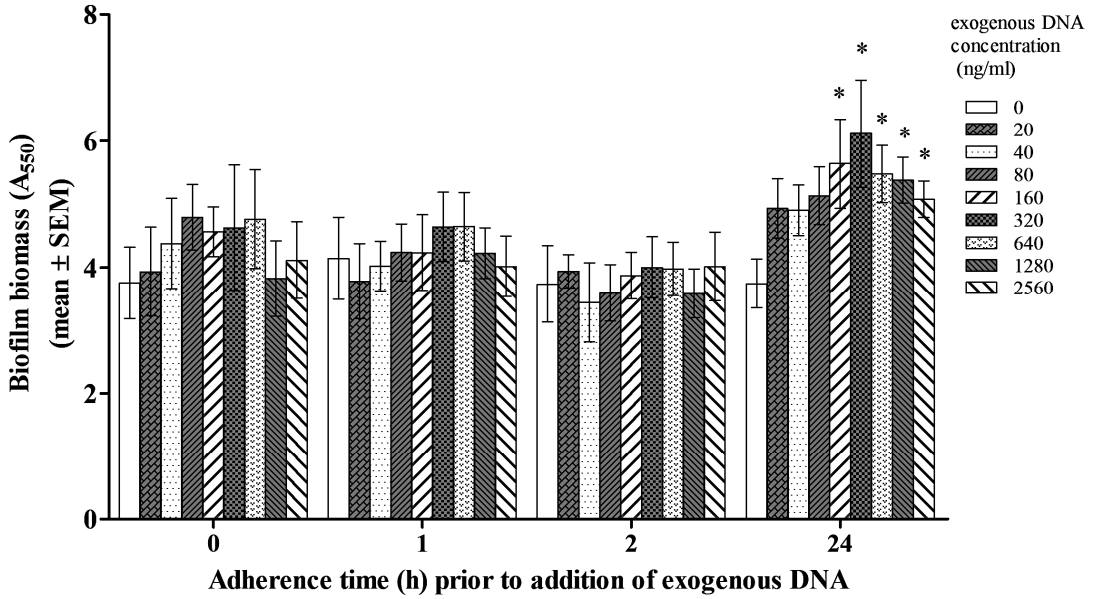

Fig.2.1-3. Effect of addition of exogenous DNA on *C. albicans* biofilm formation. Different exogenous DNA concentrations (0, 20, 40, 80, 160, 320, 640, 1280, and 2,560 ng/ml) were added to *C. albicans* cells at different times (0, 1, 2, and 24 h) of incubation in the wells of microtiter plates and incubated at 37°C under static conditions. The extent of biofilm formation was estimated by the crystal violet assay. Presented values are mean A_{550} ± SEM of four independent experiments with three to eight replicates. Statistically significant differences (compared to biofilms formed in the absence of exogenous DNA) are indicated with an asterisk. (*, $P < 0.05$).

DISCUSSION

In recent years there has been an increasing interest in the natural sources and target effects of the nucleic acids found outside the cells in several biological systems [20]. However, the study of eDNA in *C. albicans* biofilms has not been the subject of extensive research. Here, a series of experiments were designed to specifically address the contribution of eDNA to *C. albicans* biofilms ECM.

For this, we first determined the eDNA content from the ECM of 48-h biofilms using three different culture media, to ensure that eDNA is a component of ECM under different environmental growth conditions. Biofilms were developed in a flow model due to the predicted higher amounts of exopolymeric material that could be obtained when compared to biofilms developed under static conditions [15, 21]. Regardless of the growth medium used, eDNA was found to be a component of *C. albicans* ECM in all conditions used (Tables 2.1-1 and 2.1-2). In this study, it was noticed that the amount of eDNA varied considerably with the growth medium

used, with RPMI favouring the accumulation of eDNA in the ECM of *C. albicans* biofilms (Table 2.1-1). As the DNA content in the effluent medium was not determined it cannot be discarded that biofilm cells grown in YPD released more eDNA compared with RPMI but the ECM amount/ composition of RPMI biofilms promotes the accumulation of DNA. It should be noted that the association between ECM eDNA/protein content and growth medium was observed for biofilms formed in flow model.

The release of eDNA into the extracellular medium is not biofilm cells specific. It has been previously reported the release of DNA into the supernatant of *C. albicans* planktonic cells grown in RPMI and Hanks balanced salt solution [18]. The highest levels of eDNA attained in the stationary phase of growth have been suggested to result from cell death due to the nutrient depletion and toxic metabolites accumulation [18]. We also determined the amount of free DNA in the supernatant of 48-h grown *C. albicans* planktonic cultures and observed the following hierarchy of eDNA accumulation relative to the amount of protein: YPD > RPMI > YNB (unpublished data).

In bacteria, it was proposed that quorum sensing is one of the mechanisms that contributes to increased release of eDNA into the environment [22]. Recently, the eDNA content of *C. albicans chk1/chk1* ECM was determined [14], which is a mutant strain known to be nonresponsive to the *C. albicans* quorum sensing molecule farnesol [23]. The ECM of the mutant strain contained only slightly lower eDNA and protein levels/ biofilm wet weight in comparison with the wild-type strain [14], suggesting that DNA release by *C. albicans* biofilm cells may not be a controlled step linked to cell-cell communication through farnesol.

Regardless the factors that promote eDNA accumulation it was important to address the role of this ECM component in *C. albicans* biofilms. Circumstantial evidences have shown that DNA may play a structural role in *C. albicans* 48-h biofilms [13]. In this sense, we evaluated the impact of eDNA removal by DNase (Fig.2.1-1), and addition of exogenous DNA (Fig.2.1-3) at different stages of *C. albicans* biofilm formation. For the determination of the effect of these treatments on biofilms, a rapid and robust method, such as the 96-well plate model [24] that allows testing of multiple parameters in the same experiment was preferred. Furthermore, RPMI medium was the choice medium for the following experiments because it provides an optimal pH for DNase activity. Results from these series of experiments demonstrate that eDNA is an important component of *C. albicans* mature biofilms contributing to its maintenance and

stability, but is not required for the establishment of biofilms or during the very early phases of biofilm development (Fig.2.1-1 and 2.1-3).

Although it has not been determined how biofilms grown in 96-well plates compare to those developed under flow conditions the conjugation of the data obtained from the two models is consistent with eDNA being a key element of the ECM in mature *C. albicans* biofilms and playing a predominant role in biofilm structural integrity and maintenance. However, we note that eDNA role in *Candida* biofilms may be species/strain dependent since a previous study reported that *C. tropicalis* biofilm biomass is not affected by 50 μg/ml DNase treatment [13]. Data presented in this work also points to a differential role of eDNA among *C. albicans* and bacterial biofilms. In fact, for several bacterial species eDNA is required for the initial attachment as well as for subsequent early phases of biofilm formation, but only for some species DNA was found to be an important structural component of mature biofilms [8, 9].

In the last years several lines of evidence suggested that in vivo eDNA has a functional activity in the regulation of host immune response. On one hand, it was demonstrated that the administration of *C. albicans* DNA to mice at specific time points prior to infection promoted mice survival [25, 26]. On the other hand, it was demonstrated that after *C. albicans* infection of nonneutropenic rabbits fungal eDNA was detected in the plasma within the first 24 h and increased over the course of disease [18]. This eDNA may be available for interaction with Toll-like receptor 9 (TLR9), an intracellular fungal DNA receptor, as evidenced by studies showing *C. albicans* eDNA activation of bone marrow-derived myeloid dendritic cells through the TLR9/ Myeloid differentiation factor 88 signalling pathway [27], and the increased survival of TLR9 deficient mice upon challenge with *C. albicans* hyphae [28]. We can hypothesize that eDNA present on *C. albicans* biofilms may contribute to the modulation of host immune response.

Overall, this work reveals novel aspects of the role of eDNA on *C. albicans* biofilms, and properties of biofilm ECM that contribute to a better understanding of *C. albicans* biofilm lifestyle.

REFERENCES

1. Nobile, C. J. & Mitchell, A. P. (2007) Microbial biofilms: e pluribus unum. *Curr Biol* **17**, R349-R353.
2. Flemming, H. C., Neu, T. R., & Wozniak, D. J. (2007) The EPS matrix: the "house of biofilm cells". *J Bacteriol* **189**, 7945-7947.

3. Allesen-Holm, M., Barken, K. B., Yang, L. *et al.* (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* **59**, 1114-1128.
4. Vilain, S., Pretorius, J. M., Theron, J., & Brozel, V. S. (2009) DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilm. *Appl Environ Microbiol* **75**, 2861-2868.
5. Tetz, G. V., Artemenko, N. K., & Tetz, V. V. (2009) Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob Agents Chemother* **53**, 1204-1209.
6. Steinberger, R. E. & Holden, P. A. (2005) Extracellular DNA in single- and multiple-species unsaturated biofilms. *Appl Environ Microbiol* **71**, 5404-5410.
7. Bockelmann, U., Janke, A., Kuhn, R. *et al.* (2006) Bacterial extracellular DNA forming a defined network-like structure. *FEMS Microbiol Lett* **262**, 31-38.
8. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**, 1487.
9. Izano, E. A., Amarante, M. A., Kher, W. B., & Kaplan, J. B. (2008) Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl Environ Microbiol* **74**, 470-476.
10. Mulcahy, H., Charron-Mazenod, L., & Lewenza, S. (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* **4**, e1000213.
11. Ramage, G., Saville, S. P., Thomas, D. P., & Lopez-Ribot, J. L. (2005) *Candida* biofilms: an update. *Eukaryot Cell* **4**, 633-638.
12. Nobile, C. J., Nett, J. E., Hernday, A. D. *et al.* (2009) Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* **7**, e1000133.
13. Al-Fattani, M. A. & Douglas, L. J. (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**, 999-1008.
14. Paramonova, E., Krom, B. P., van der Mei, H. C., Busscher, H. J., & Sharma, P. K. (2009) Hyphal content determines the compression strength of *Candida albicans* biofilms. *Microbiology* **155**, 1997-2003.
15. Uppuluri, P., Chaturvedi, A. K., & Lopez-Ribot, J. L. (2009) Design of a simple model of *Candida albicans* biofilms formed under conditions of flow: development, architecture, and drug resistance. *Mycopathologia* **168**, 101-109.
16. Thomas, D. P., Bachmann, S. P., & Lopez-Ribot, J. L. (2006) Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. *Proteomics* **6**, 5795-5804.
17. Qin, Z., Ou, Y., Yang, L. *et al.* (2007) Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* **153**, 2083-2092.
18. Kasai, M., Francesconi, A., Petraitiene, R. *et al.* (2006) Use of quantitative real-time PCR to study the kinetics of extracellular DNA released from *Candida albicans*, with implications for diagnosis of invasive candidiasis. *J Clin Microbiol* **44**, 143-150.
19. Fowler, J. & Cohen, L. (1995) *Statistics for ornithologists*. 2nd ed. British Trust for Ornithology.
20. Vlassov, V. V., Laktionov, P. P., & Rykova, E. Y. (2007) Extracellular nucleic acids. *Bioessays* **29**, 654-667.

21. Hawser, S. P., Baillie, G. S., & Douglas, L. J. (1998) Production of extracellular matrix by *Candida albicans* biofilms. *J Med Microbiol* **47**, 253-256.
22. Spoering, A. L. & Gilmore, M. S. (2006) Quorum sensing and DNA release in bacterial biofilms. *Curr Opin Microbiol* **9**, 133-137.
23. Kruppa, M., Krom, B. P., Chauhan, N., Bambach, A. V., Cihlar, R. L., & Calderone, R. A. (2004) The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot Cell* **3**, 1062-1065.
24. Pierce, C. G., Uppuluri, P., Tristan, A. R. *et al.* (2008) A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc.* **3**, 1494-1500.
25. Dimitrova, P., Yordanov, M., Danova, S., & Ivanovska, N. (2008) Enhanced resistance against systemic *Candida albicans* infection in mice treated with *C. albicans* DNA. *FEMS Immunol Med Microbiol* **53**, 231-236.
26. Yordanov, M., Dimitrova, P., Danova, S., & Ivanovska, N. (2005) *Candida albicans* double-stranded DNA can participate in the host defense against disseminated candidiasis. *Microbes Infect* **7**, 178-186.
27. Miyazato, A., Nakamura, K., Yamamoto, N. *et al.* (2009) Toll-like receptor 9-dependent activation of myeloid dendritic cells by Deoxynucleic acids from *Candida albicans*. *Infect Immun* **77**, 3056-3064.
28. Bellocchio, S., Montagnoli, C., Bozza, S. *et al.* (2004) The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol* **172**, 3059-3069.

Chapter 2.2 Addition of DNase improves the in vitro activity of antifungal drugs against *Candida albicans* biofilm

Abstract

Cells within *Candida albicans* biofilms display decreased susceptibility to most clinically used antifungal agents. We recently demonstrated that extracellular DNA plays an important role in biofilm integrity, as a component of the biofilm matrix. The main objective of this work was to gain insight into the contribution of extracellular DNA to *C. albicans* biofilms antifungal susceptibility by the investigation of the impact of the combined use of deoxyribonuclease I (DNase) and antifungals to treat biofilms. Here we report the improved in vitro efficacy of DNase in combination with amphotericin B and caspofungin, but not fluconazole, against *C. albicans* biofilms. This work suggests that agents that target processes affecting the biofilm structural integrity may have potential use as a therapeutic adjuvant in the treatment of biofilm-associated candidiasis.

The results presented over this chapter were:

(i) submitted as,

Martins, M., Henriques, M. Lopez-Ribot, J.L., Oliveira, R. Addition of DNase improves the in vitro activity of antifungal drugs against *Candida albicans* biofilms.

(ii) presented as poster communication,

Martins, M., Uppuluri, P., Thomas, D.P., Cleary, I.A. Henriques, M. Lopez-Ribot, J.L.,Oliveira, R. 2010. Game and player: *Candida albicans* biofilm lifestyle and extracellular DNA. **10th ASM Conference on *Candida* and Candidiasis**, Miami, March 22nd – 26th. Book of Abstracts. 250B. p. 153.

INTRODUCTION

Candida albicans is an opportunistic fungal organism that is present as part of the normal microbiota of healthy individuals. However, when the host immune system is impaired or the competing bacterial microbiota is altered, *C. albicans* can overwhelm the host defences and gain access to the bloodstream, invading tissues, or contaminating medical devices, causing life threatening infections [1]. These infections are often associated with the formation of biofilms, and one of the major concerns with disease management is the fact that *C. albicans* biofilm cells display reduced susceptibility against azoles and polyenes in comparison to planktonic cells. This intrinsic property of biofilms is likely to be multifactorial and has been associated with factors such as cells physiological state, activation of drug efflux pumps, and a protective effect of the extracellular matrix (ECM) [2]. Recently our group showed that extracellular DNA (eDNA) is a critical component of the *C. albicans* biofilm ECM that contributes to biofilm structural integrity [3].

In order to gain further insight into the contribution of eDNA to *C. albicans* biofilms antifungal susceptibility, in the present study we have investigated the impact of the combined use of deoxyribonuclease I (DNase) (which degrades eDNA) and antifungals to treat *C. albicans* biofilms.

MATERIAL AND METHODS

Chemicals

A polyene (amphotericin B), echinocandin (caspofungin), and azole (fluconazole) were chosen for their different mechanisms of action. Drug solutions were prepared according to the Clinical and Laboratory Standards Institute recommendations, and the range of concentrations were as follows: amphotericin B (Sigma, St. Louis, MO), 0.06 to 16 mg/l; caspofungin (Merck, NJ, USA), 0.008 to 2 mg/l, and fluconazole (Pfizer, CT, USA), 4 to 1,024 mg/l. DNase I from bovine pancreas (Sigma) stock solutions were prepared in saline supplemented with 5 mM of $MgCl_2$ (Merck, PA, USA). The enzyme, which retains exonuclease activity in nutrient media for 24 h [4], was used at 0.13 mg/ml, concentration previously shown to decrease *C. albicans* biofilm biomass [3].

Biofilm formation and antifungal susceptibility assay

C. albicans SC5314 biofilms were formed in 96-well microtiter plates as previously described, using Roswell Park Memorial Institute 1640 (RPMI) medium (Mediatech Inc., Herdon, VA, USA) buffered with 3-[N-morpholino] propanesulfonic acid (Research Products International Corp Mount Prospect, IL, USA) to pH 7 [5]. Biofilms (24-h) were washed with phosphate buffered saline (PBS) (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma) and challenged with RPMI containing the different concentrations of antifungals alone or in combination with DNase. Control biofilms were challenged with RPMI, RPMI $MgCl_2$, and RPMI DNase. Following 24-h incubation at 37°C, biofilm cells were washed with PBS and mitochondrial activity estimated by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assay [5]. Sessile minimum inhibitory concentrations (SMIC) were determined at 50% (SMIC50) and 80% (SMIC80) decrease in absorbance at 490 nm, in comparison with the corresponding control biofilms challenged with RPMI for antifungals tested alone or challenged with RPMI DNase for antifungals tested in combination with DNase.

Reproducibility and statistical analyses

All the experiments were repeated on three separated occasions with eight replicates each. Statistical analyses were performed using GraphPad statistical software (GraphPad Software, San Diego, CA, USA). The normality of the data was examined using D'Agostino and Pearson omnibus normality test. To determine if the DNase improves the in vitro efficacy of antifungal drugs against *C. albicans* biofilms, the percentage of colorimetric readings for XTT reduction assays were analysed using two-way ANOVA followed by Bonferroni post-hoc tests. Statistical significance was defined as $P< 0.05$.

Results and Discussion

Consistent with previous investigations [6, 7], results revealed that the amphotericin B SMIC80 (1 mg/l) (Fig.2.2-1 *a*) is within the resistance range of this drug (> 1 mg/l). However, addition of DNase to amphotericin B resulted in SMIC50 (0.06 mg/l) and SMIC80 (0.25 mg/l) values

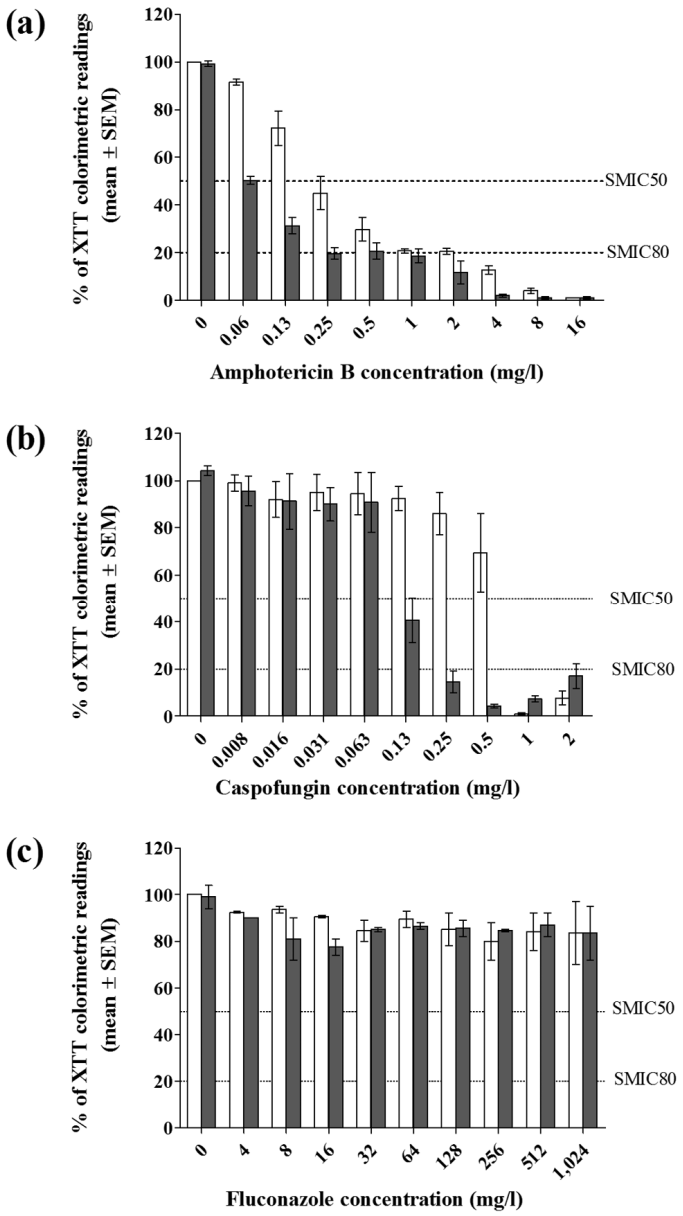

Fig.2.2-1. Activity of amphotericin B (***a***), caspofungin (***b***), and fluconazole (***c***) against *C. albicans* SC5314 24-h biofilms used alone (white bars) or in combination with DNase (black bars). Data is presented as mean ± standard error of mean (SEM) of the percentage of colorimetric readings for XTT reduction assays (% of XTT colorimetric readings), as compared with drug-free controls. DNase at 0.13 mg/ml was added together with serial dilutions of antifungal agents. SMIC are indicated.

that were two dilutions lower than when this polyene agent was used alone (Fig.2.2-1 *a*). Accordingly, between the amphotericin range of 0.06 and 0.25 mg/l the % of XTT colorimetric readings were significantly lower when the antifungal drug was used in combination with DNase than when used alone. Importantly, the SMIC50 values in the presence of DNase (Fig.2.2-1 *a*)

were below the planktonic minimal inhibitory concentration (typically 0.25 mg/l) [6], and the SMIC80 values for amphotericin B plus DNase (Fig.2.2-1 *a*) were below the resistance range for this antifungal agent.

As reported before [8-10], *C. albicans* biofilm cells were susceptible to caspofungin, with the SMIC80 value (1 mg/l, Fig.2.2-1 *b*) within the susceptible range (≤ 2 mg/l). Our results indicate that, similarly to amphotericin B, the in vitro activity of caspofungin against *C. albicans* biofilms was increased when used in combination with DNase (Fig.2.2-1 *b*). In fact, SMIC50 and SMIC80 values for caspofungin plus DNase were three dilutions (0.13 mg/l) and two dilutions (0.25 mg/l) lower, respectively, when compared with biofilms exposed to this antifungal alone (Fig.2.2-1 *b*). Therefore, biofilm cells treated with DNase and caspofungin ranging from 0.13 to 0.5 mg/l exhibited lower % of XTT readings in comparison with those treated with caspofungin only ($P < 0.05$). Of note, trailing growth with caspofungin is usually observed [11], and this was not abolished with the addition of DNase (Fig. 2.2-1 *b*). However, to confirm the trailing growth effect, higher caspofungin concentrations should be evaluated.

It is well established in the field that cells within biofilms are intrinsically less susceptible to azole derivatives [6, 9, 10]. Our results for *C. albicans* biofilm cells reduced susceptibility to fluconazole (Fig.2.2-1 *c*) show that both SMIC80 and SMIC50 values are higher than 1,024 mg/l. Contrary to the observed for the other drugs tested, the susceptibility of biofilm cells against fluconazole was not changed by the addition of DNase (Fig.2.2-1 *c*). Even though, other azole drugs need to be tested.

Several literature reports have shown that eDNA contributes to bacterial biofilms increased susceptibility to some antibiotics [12, 13], sodium dodecyl sulfate [14], antiseptics, and disinfectants agents [15]. It has been suggested that this effect may be due to eDNA induction of genes involved in the modification of lipopolysaccharides [13] and/or poor penetration of drugs within biofilms [12]. However, this is probably not a biofilm specific trait, as there is some controversy on the effect of DNase on the antimicrobial susceptibility of bacterial planktonic cells [4, 16]. In addition exogenous DNA was shown to promote its increase [13]. We observed that DNase increases *C. albicans* planktonic cells susceptibility to amphotericin B (by

one dilution) and caspofungin (by three dilutions), but not to fluconazole, in comparison with cells challenged only with the drugs (data not shown). In contrast, in *C. albicans* biofilm and planktonic cultures treated with DNA (320 ng/ml) and antifungals the drug susceptibility was not affected, when compared to antifungals tested alone (data not shown). Overall, these results suggest that eDNA, as a component of biofilm ECM, may not impede antifungals diffusion through biofilms, in accordance with previous reports showing that *C. albicans* biofilm ECM do not impair antifungals penetration [17]. Nevertheless, products of the enzymatic digestion of eDNA or DNase might interact with cell component(s) facilitating the action of antifungal agents. In fact, Nett et al. [18] have shown shown that ECM β-glucans are involved in biofilm antifungal resistance by hampering the access of the drugs access to their targets inside the cells.

In conclusion, our results show that *C. albicans* biofilm cells susceptibility to amphotericin B and caspofungin is enhanced by the addition of DNase. Although the mechanism(s) and in vivo efficacy of this effect needs to be further explored, this work suggests that agents that target processes affecting the biofilm structural integrity may have potential use as a therapeutic adjuvants in the treatment of biofilm-associated candidiasis, at least as part of devices antifungal lock therapy.

REFERENCES

1. d'Enfert, C. (2009) Hidden killers: persistence of opportunistic fungal pathogens in the human host. *Curr Opin Microbiol* **12**, 358-364.
2. Seneviratne, C. J., Jin, L. J., Samaranayake, Y. H., & Samaranayake, L. P. (2008) Cell density and cell aging as factors modulating antifungal resistance of *Candida albicans* biofilms. *Antimicrob Agents Chemother* **52**, 3259-3266.
3. Martins, M., Uppuluri, P., Thomas, D. P. *et al.* (2010) Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia* **169**, 323-331.
4. Tetz, V. V. & Tetz, G. V. (2010) Effect of extracellular DNA destruction by DNase I on characteristics of forming biofilms. *DNA Cell Biol.***in press**
5. Pierce, C. G., Uppuluri, P., Tristan, A. R. *et al.* (2008) A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc* **3**, 1494-1500.

6. Ramage, G., Vande Walle, K., Wickes, B. L., & Lopez-Ribot, J. L. (2001) Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* **45**, 2475-2479.
7. Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., & Ghannoum, M. A. (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**, 5385-5394.
8. Bachmann, S. P., Vandewalle, K., Ramage, G. *et al.* (2002) In vitro activity of caspofungin against *Candida albicans* biofilms. *Antimicrob Agents Chemother.* **46**, 3591-3596.
9. Kuhn, D. M., George, T., Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2002) Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother.* **46**, 1773-1780.
10. Ramage, G., Vandewalle, K., Bachmann, S. P., Wickes, B. L., & Lopez-Ribot, J. L. (2002) In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Chemother.* **46**, 3634-3636.
11. Wiederhold, N. P. (2007) Attenuation of echinocandin activity at elevated concentrations: a review of the paradoxical effect. *Curr Opin Infect Dis* **20**, 574-578.
12. Tetz, G. V., Artemenko, N. K., & Tetz, V. V. (2009) Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob Agents Chemother* **53**, 1204-1209.
13. Mulcahy, H., Charron-Mazenod, L., & Lewenza, S. (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* **4**, e1000213.
14. Allesen-Holm, M., Barken, K. B., Yang, L. *et al.* (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* **59**, 1114-1128.
15. Kaplan, J. B. (2009) Therapeutic potential of biofilm-dispersing enzymes. *Int J Artif Organs* **32**, 545-554.
16. Vilain, S., Pretorius, J. M., Theron, J., & Brozel, V. S. (2009) DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilm. *Appl Environ Microbiol* **75**, 2861-2868.
17. Al-Fattani, M. A. & Douglas, L. J. (2004) Penetration of *Candida* biofilms by antifungal agents. *Antimicrob Agents Chemother* **48**, 3291-3297.
18. Nett, J. E., Sanchez, H., Cain, M. T., & Andes, D. R. (2010) Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J Infect Dis* **202**, 171-175.

CHAPTER 3

Insights into *Candida* world: extracellular alcohols

Chapter 3.1 Effect of farnesol in Non–*Candida albicans Candida* species

Abstract

Non-*Candida albicans Candida* (NCAC) species are emerging as significant pathogens. The present study aimed to investigate the effect of *E*,*E*-farnesol (farnesol) (*Candida albicans* signaling molecule) in *Candida glabrata*, *Candida krusei*, and *Candida tropicalis* planktonic cells. Stationary cultures challenged with several farnesol concentrations (5 to 150 μM) were monitored in terms of morphology, cell growth, viability, and cell cycle progression. Farnesol did not induce morphological changes in the assayed NCAC species. Additionally, changes in NCAC species growth were observed: (i) farnesol (100 and 150 μM) compromised *C. glabrata* growth, which was not associated with changes in cell survival, although S-G2/M cell cycle phase transition was delayed; (ii) farnesol (50, 100, and 150 μM) delayed *C. krusei* growth without compromising cell viability, but delaying G0/G1 cell cycle phase; (iii) farnesol 100 and 150 μM delayed *C. tropicalis* growth, that was associated with a decrease in cell viability and G0/G1 phase cell cycle phase delay. The present findings suggest that farnesol impairs NCAC species but the mode of action of this molecule is species dependent.

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(i) presented as poster communication,

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Martins, M., Henriques, M., Azeredo, J., Oliveira, R. 2007. Farnesol beyond morphogenesis control: effect in Non-*Candida albicans Candida* species. **Second FEBS Advanced Lecture Course Human Fungal Pathogens**. La Colle sur Loup, France, May 11th – 17th. Book of Abstracts, P18, p. 74.

INTRODUCTION

Candida albicans is the predominant etiological agent of candidiasis, although Non-*C. albicans Candida* (NCAC) species are emerging as significant pathogens [1]. NCAC species tend to be less susceptible to the commonly used antifungals than *C. albicans,* and are frequently associated with host diminished defenses and implanted devices [2]. As *C. albicans* has been for many years the focus of investigators', the research on NCAC species has relied on clinical laboratory studies, hampering the knowledge on the biology of these species.

E,*E*-Farnesol (farnesol) is a molecule produced by *C. albicans*, which depending on the experimental conditions used, can regulate yeast morphogenesis [3], biofilm formation [4], or act as an apoptotic effector [5]. Additionally, for other yeast and fungi such as *Saccharomyces cerevisiae* [6], *Aspergillus nidulans* [7], *Candida dubliniensis* [8], and *Candida parapsilosis* [9] farnesol has been described as a growth inhibitor or cell death effector. In *C. albicans*, farnesol mediated cell death correlated with mitochondrial degradation, reactive oxygen species accumulation, and caspases activation [5]. In *C. parapsilosis*, farnesol induced growth delay was associated with changes in lipid metabolism, ribosome biogenesis, and amino acid biosynthesis gene expression [9]. In *C. dubliniensis*, farnesol promoted cell death that was associated with cell membrane disruption [8]. However, these distinct mechanisms induced by farnesol cannot be associated with a species specific effect due to the different experimental conditions used in the independent studies.

To date, the effect of farnesol in other NCAC species has not been reported, thus, the objective of this work was to evaluate the effect of this molecule in *Candida glabrata*, *Candida krusei*, and *Candida tropicalis* cells in terms of morphology, growth, viability, and cell cycle progression.

MATERIAL AND METHODS

Strains and culture conditions

C. glabrata ATCC 2001, *C. krusei* ATCC 6258, and *C. tropicalis* ATCC 750 were used in this study. *C. parapsilosis* ATCC 22019 growth was already described to be regulated by farnesol

[9], thus, this strain was used as a control. *Candida* species cells were propagated onto Sabouraud dextrose agar medium (Liofilchem) and incubated at 37°C for 24 h. Batches of Sabouraud dextrose broth (Liofilchem) (20 ml in 50 ml flasks) were inoculated with freshly grown *Candida* cells, and incubated at 37°C, 130 rpm, for 24 h. After that, cells were pelleted, washed with water, and enumerated in a hematocytometer. Before use in further experiments, 1×10^6 cells/ml standardized suspensions (20 ml in 125 ml flasks) were prepared in Roswell Park Memorial Institute 1640 (RPMI) (Sigma), supplemented with sodium bicarbonate (Pronolab) 2.0 g/l, and buffered with 3-[N-morpholino] propanesulfonic acid (Sigma) (0.165 M) to pH 7.0.

Chemicals

Farnesol (Sigma) stock solutions were freshly prepared in methanol before each experiment. The pure compound was stored according to the supplier's specifications. Working solutions of farnesol (5, 50, 100, and 150 μM) were prepared in RPMI medium. Controls included methanol (vehicle) and octylphenol ethylene oxide condensate (Triton X-100; Sigma) at the maximum concentration used in the farnesol experiments: 0.07% (v/v) and 150 μM respectively.

Morphology assessment

To evaluate the effect of farnesol on *Candida* morphology, standardized cells suspensions were grown at 37°C, 130 rpm in RPMI medium containing farnesol from 0 to 150 μM or vehicle, for 2 h. Afterwards, samples (1 ml) were collected, washed with water, and fixed with 70% ethanol (v/v) at 4°C. After 24 h, cells were pelleted, ressuspended in water, and observed by light microscopy. Experiments were performed on three separate occasions.

Growth assessment

To study the effect of farnesol on *Candida* growth, standardized cells suspensions were grown at 37°C, 130 rpm in RPMI medium containing the indicated farnesol concentrations (0 to 150 μM). Appropriated controls were included in each assay. Growth was monitored at 0, 2, 4, 6, 8, and 24 h. First, absorbance at 640 nm (A_{640}) (Bio-Tek Synergy HT, Izasa) was determined. Second, the total cell number was determined after counting cells in a haemocytometer. The

percentage of growth inhibition was calculated as described by Rossignol *et al.* [9]: [1-($\Delta_{farnesol}/\Delta_{vehicle}$)] × 100, where Δ is the difference between the number of cells at a specific time point after addition of farnesol and that at the time of addition. Experiments were performed in duplicate, with at least three independent replicates.

At selected time points, samples exhibiting a decrease in growth and the corresponding controls (farnesol 0 μM and vehicle) were used for viability assessments and DNA content analysis (see below).

Viability assessment

To evaluate the effect of farnesol on *Candida* viability, at time 0 h and 2 h of farnesol exposure, cells from selected cultures were pelleted and washed with water. First, colony forming ability was determined by plating appropriate dilutions on Sabouraud dextrose agar. After 48 h of incubation at 37°C, colony forming units (CFU) were counted. The percentage of survival was calculated as [(CFU at time 2 h/ CFU at time 0 h) × 100]. Second, cell membrane integrity was assessed after staining with 0.4% trypan blue (Sigma) [10]. Cell suspensions (100 μl) were mixed with an equal volume of dye and incubated at room temperature for three minutes. Cells with intact membranes exclude trypan blue and cells that exhibit membrane damages are labelled with the dye. The number of cells excluding or not trypan blue was enumerated in a haemocytometer. The percentage of trypan blue negative cells was calculated as the [(number of trypan negative cells/ number total cells) × 100]. All the assays were performed on three separate occasions with two replicates.

DNA content analysis

To evaluate the effect of farnesol on *Candida* cell cycle, at selected time points DNA content was analysed using SYBR Green I, as previously described [11]. Samples (1 ml) were pelleted and fixed overnight with 70% ethanol (v/v) at 4°C. Cell suspensions were pelleted, washed, and resuspended in 50 mM sodium citrate (Sigma) buffer, pH 7.5, to a final cell concentration of 5 × 10^6 cells/ml. Cells were subjected to 3 consecutive ultrasound pulses at 40 W for 1 s, with an interval of 1 to 2 s between each pulse (Ultrasonic Processor, Cole-Parmer). Then, cell suspensions were treated with 125 μg ribonuclease A (Invitrogen) for 60 min at 50°C, followed

by a 0.5 mg proteinase K treatment for 60 min at 50°C. Cell staining was achieved by overnight incubation with 25 × SYBR Green I (Molecular Probes) final concentration, at 4°C. Before analysis by flow cytometry, Triton X-100 to a final concentration of 0.25% (v/v) was added to each sample. Analyses were performed on an EPICS XL-MCL (Beckman-Coulter Corporation) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Offline data was analyzed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0, and Expo32 ADC software. Two independent sets of cells were analysed.

Microscopy analyses

Light microscopy analyses of cell morphology and cell membrane integrity were carried out on an Olympus BX51 microscope fitted with a 40 × objective and equipped with a DP71 camera.

Statistical analyses

Results are expressed as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism, version 5.00 software for Windows (GraphPad Software). Comparisons between the number cells/ml (farnesol treatment vs control) were performed by two-tailed unpaired t-test (confidence interval 95%). Comparisons between the: (i) distributions of the percentage of survival, (ii) trypan blue negative cells, and (iii) single cells in farnesol treatments vs control were performed by two-tailed Mann-Whitney test (confidence interval 95%). Differences were considered significant at $P < 0.05$.

RESULTS

Under the conditions used in this study, farnesol 50, 100, and 150 μM delayed growth of *C. parapsilosis* stationary cells transferred to fresh medium, when compared to controls, similarly to previously described [9]. For all the experiments performed, no differences were observed between farnesol 0 μM and vehicle or Triton X-100 treatments. Therefore, only data from vehicle assays are presented.

Effect of farnesol in *C. glabrata*

C. glabrata morphology analysis (Fig.3.1-1 *A*) revealed that cells grown in the presence of vehicle (Fig.3.1-1 *A-I*) or farnesol- exemplified for 5 μM (Fig.3.1-1 *A-II*) and 150 μM farnesol (Fig.3.1-1 *A-III*) - exhibited a yeast form.

Growth monitoring assays (Fig.3.1-1 *B* and *C*) showed that farnesol 5 and 50 μM did not affect *C. glabrata* cell growth vs vehicle. The growth of cells exposed to 100 and 150 μM farnesol was indistinguishable from that of controls from 0 to 4 h in terms of cell density (Fig.3.1-1 *C*), although it was lower than that of vehicle at 6 and 8 h when compared to controls (Fig.3.1-1 *C*) ($P< 0.05$). The maximum reduction in growth was observed at 8 h, specifically, 45 and 36% for 100 and 150 μM treated cultures, respectively. The cells recovered from the farnesol mediated growth arrest, although after 24 h exposure to 150 μM farnesol, cell density was lower than that of vehicle (Fig.3.1-1 *C*) ($P< 0.05$).

C. glabrata viability, assessed after farnesol addition (Table 3.1-1) revealed that farnesol 100 and 150 μM did not affect the ability of cells to form colonies, or cell membrane integrity, as indicated by the similar proportions of survival and trypan blue negative cells, respectively (Table 3.1-1).

Consistent with the growth delay profile induced by farnesol (Fig.3.1-1 *C*), from 0 to 2 h, the emergence of cellular buds was not affected by the compound treatment, as indicated by the similar percentages of single cells at 2 h for treated and untreated cultures (Fig.3.1-1 *D*). From 6 to 8 h the proportion of single cells for the vehicle treated cells increased (Fig.3.1-1 *D*), suggesting the entry of cells in the late log phase, with a return of cells to G1 phase and bud separation. In contrast, in the same time interval, the percentage of single cells in the farnesol treated cultures remained unaltered (Fig.3.1-1 *D*). As a consequence, in farnesol (100 and 150 μM) treated cells the proportion of single cells at 8 h of growth was lower than that of control ($P< 0.05$). This was confirmed by the lower percentage of cells in the G0/G1 phase of the cell cycle at this time point. Specifically, the percentage of cells in G0/G1 population for untreated cells was 81% vs 38% for 100 and 150 μM treatments, respectively.

These data show that farnesol does not affect *C. glabrata* morphology, but at concentrations ≥ 100 μM delays cell growth, increasing the length of S-G2/M cell cycle phases.

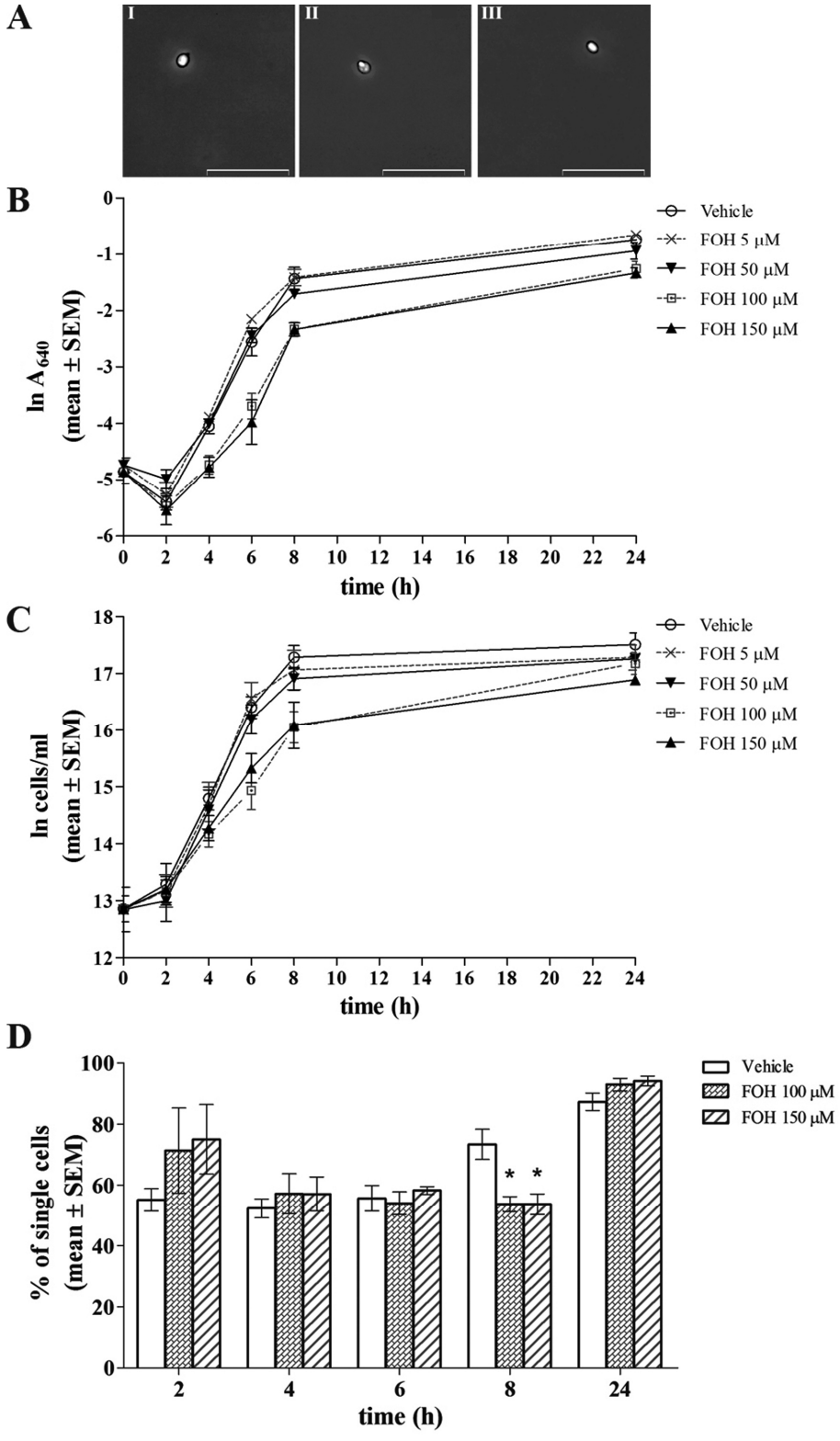

Fig.3.1-1. Characterization of farnesol (FOH) effect in *C. glabrata*. Morphology (***A***) 2 h after vehicle (***I***), FOH 5 µM (***II***), and FOH 150 µM (***III***) treatments is exemplified (bar = 20 µm). Growth is expressed as mean ln A_{640} ± SEM (***B***) and mean ln cells/ml ± SEM (***C***). Progression through the cell cycle is expressed as mean percentage of single cells ± SEM (***D***). At 0 h, the mean percentage of single cells was 100%. At each time point statistically significant differences (farnesol treatment) vs control are indicated with an asterisk (*, P< 0.05). For simplicity statistical significances were omitted from the panels B and C.

Table 3.1-1 *Candida* species viability 2 h after farnesol (FOH) exposure

Candida species	Viability (mean ± SEM)[a]	Vehicle	FOH 50 µM	FOH 100 µM	FOH 150 µM
C. glabrata	% Survival	122.3 ± 4.7	n.d.	107.6 ± 16.3	109 ± 10.6
	% Trypan blue negative cells	98.5 ± 0.8	n.d.	93 ± 4.1	97.3 ± 1.6
C. krusei	% Survival	81.9 ± 10.4	78 ± 7.5	72.2 ± 7.8	73.6 ± 3
	% Trypan blue negative cells	98.5 ± 1.1	87.7 ± 4.9	92.3 ± 4.1	76.7 ± 10.3
C. tropicalis	% Survival	127.6 ± 14.1	n.d.	120.5 ± 5.3	84.2 ± 0.7
	% Trypan blue negative cells	93.5 ± 3.4	n.d.	41.1 ± 9.5	51.9 ± 7.5

n.d., not determined; [a]Viability assessment was based on % survival, calculated as [(CFU at 2 h/ CFU at 0 h) × 100], and % of trypan blue negative cells, calculated as [(number of trypan blue negative cells/ number total cells) × 100]

Effect of farnesol in *C. krusei*

C. krusei morphology was evaluated after cells exposure to several farnesol concentrations. The pseudohyphal growth exhibited by *C. krusei* cells in the control conditions (Fig.3.1-2 *A-I*) was maintained upon farnesol treatments ranging from 5 (Fig.3.1-2 *A-III*) to 150 µM (Fig.3.1-2 *A-IV*).

C. krusei cell growth monitoring under the different treatments (Fig.3.1-2 *B* and *C*) indicated that farnesol 5 µM does not affect cell growth when compared to vehicle (Fig.3.1-2 *B* and *C*). However, farnesol ≥ 50 µM treatments resulted in a strong growth inhibition (Fig.3.1-2 *B* and *C*), with the maximum inhibition levels (up to 70%) observed at 4 h of growth. The cells recovered during the following hours of growth, although 24 h after farnesol (50 to 150 µM) addition the cell density of treated cultures was lower than that of controls (Fig.3.1-2 *C*) ($P < 0.05$).

C. krusei cells viability assessment (Table 3.1-1) showed that farnesol (50 to 150 µM) did not induce major changes on cell viability, as indicated by the similar percentages of survival and trypan blue negative cells for treated and untreated cells ($P > 0.05$).

The proportion of *C. krusei* single cells (Fig.3.1-2 *D*) and cellular DNA content (data not shown) were assessed in the presence of farnesol (50 to 150 µM) . The addition of farnesol 50 µM did not affect cell budding (Fig.3.1-2 *D*) or G0/G1 cell cycle phase distribution vs vehicle (data not shown). However, farnesol at 100 and 150 µM delayed cell division, as indicated by the higher percentage of single cells at 4, 6, and 8 h ($P < 0.05$, vs vehicle) (Fig.3.1-2 *D*). A comparable delay in the G1/S cell cycle phase transition was observed upon 100 and 150 µM

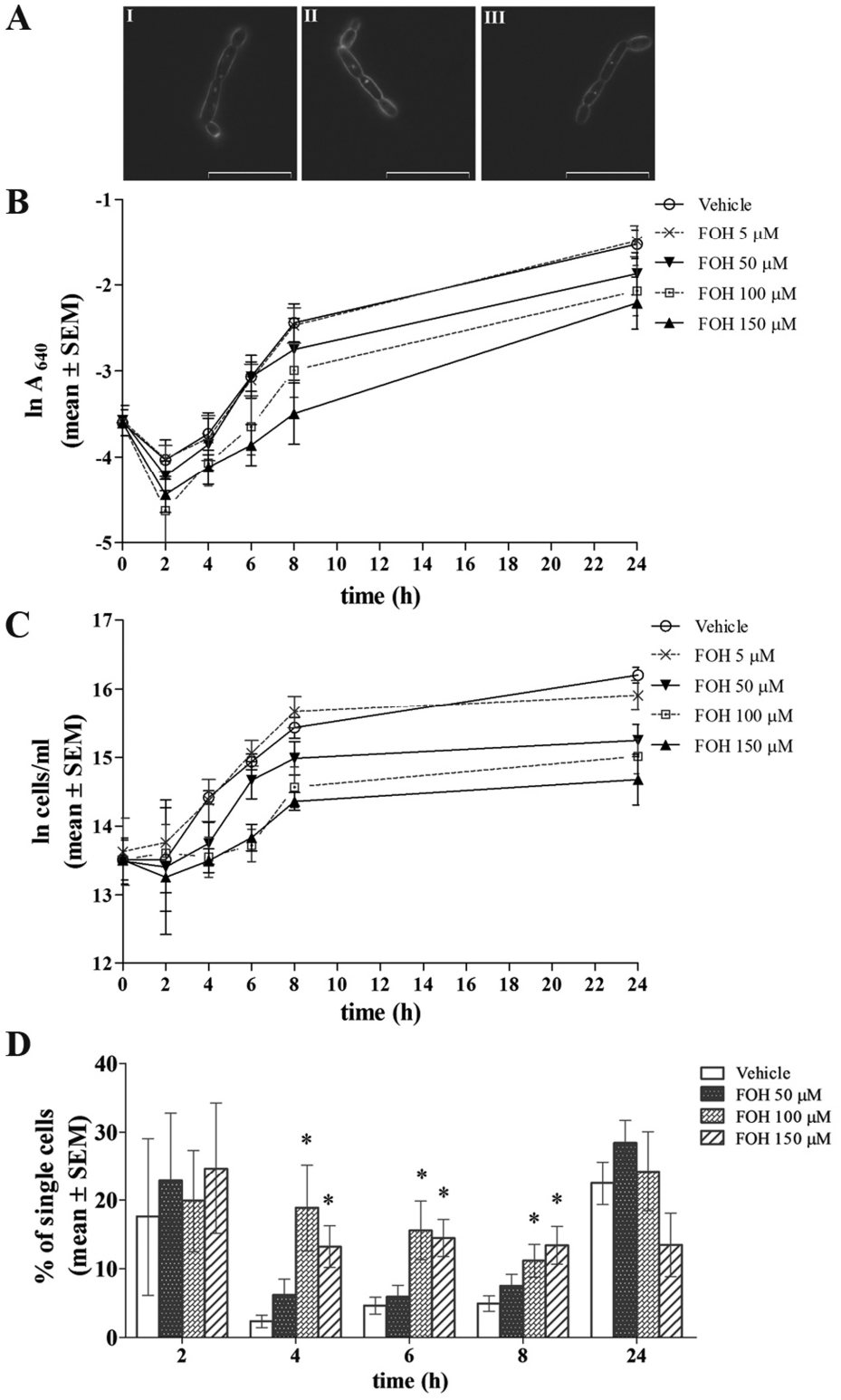

Fig.3.1-2. Characterization of farnesol (FOH) effect in *C. krusei*. Morphology (***A***) 2 h after vehicle (**I**), FOH 5 µM (***II***), and FOH 150 µM (***III***) treatments is exemplified (bar = 20 µm). Growth is expressed as mean ln A_{640} ± SEM (***B***) and mean ln cells/ml ± SEM (***C***). Progression through the cell cycle is expressed as mean percentage of single cells ± SEM (***D***). At 0 h, the mean percentage of single cells was 27%. At each time point statistically significant differences (farnesol treatment vs control) are indicated with an asterisk (*, P< 0.05). For simplicity statistical significances were omitted from the panels B and C.

farnesol treatments from 4 to 8 h (data not shown). For example, at 4 h G0/G1 cell population was 4% in the vehicle cultures vs 14% and 15% in 100 and 150 μM farnesol treated cultures, respectively.

These data show that farnesol does not regulate *C. krusei* morphology, but compromises cell growth, lengthening the G0/G1 cell cycle phase.

Effect of farnesol in *C. tropicalis*

Microscopic analysis of *C. tropicalis* revealed that the cell morphology observed in vehicle (Fig.3.1-3 *A-I*) cultures did not change upon farnesol 5 μM (Fig.3.1-3 *A-II*) or 150 μM (Fig.3.1-3 *A-III*) treatments.

C. tropicalis growth analysis did not reveal significant differences between farnesol 5 and 50 μM treated vs control cultures (Fig. 3.1-3 *B* and *C*). In contrast, *C. tropicalis* cells exposed to farnesol 100 and 150 μM displayed reduced growth (Fig.3.1-3 *B* and *C*). The maximum inhibition levels were observed at 4 h, specifically, 79% and 100% for 100 and 150 μM treatments, respectively. Farnesol treated cells recovered during the following hours of growth (Fig.3.1-3 *B* and *C*) although at 24 h, cell density was lower than that of vehicle for all farnesol concentrations tested (Fig.3.1-3 *C*) ($P< 0.05$).

C. tropicalis cell viability revealed that farnesol 150 μM induced a slight reduction in the ability of cells to form colonies (vs vehicle) (Table 3.1-1) ($P < 0.05$). In addition, farnesol 100 and 150 μM altered cell membrane permeability, as indicated by the lower percentages of trypan blue negative cells (vs vehicle) (Table 3.1-1) ($P< 0.05$).

Data from cell cycle progression of *C. tropicalis* treated cells revealed that until 6 h of growth, the percentage of single cells in farnesol 100 and 150 μM treated cultures was higher than in vehicle ($P < 0.05$) (Fig.3.1-3 *D*). This was confirmed by the delay in the G1/S cell cycle phase transition (data not shown). For example, at 6 h, the proportion of cells in the G0/G1 subpopulation was 8% for vehicle cultures vs 34% for 100 and 150 μM farnesol ones.

These data show that although farnesol does not regulate *C. tropicalis* morphology, it impairs cell growth with an involvement of cell viability and G0/G1 cell cycle progression.

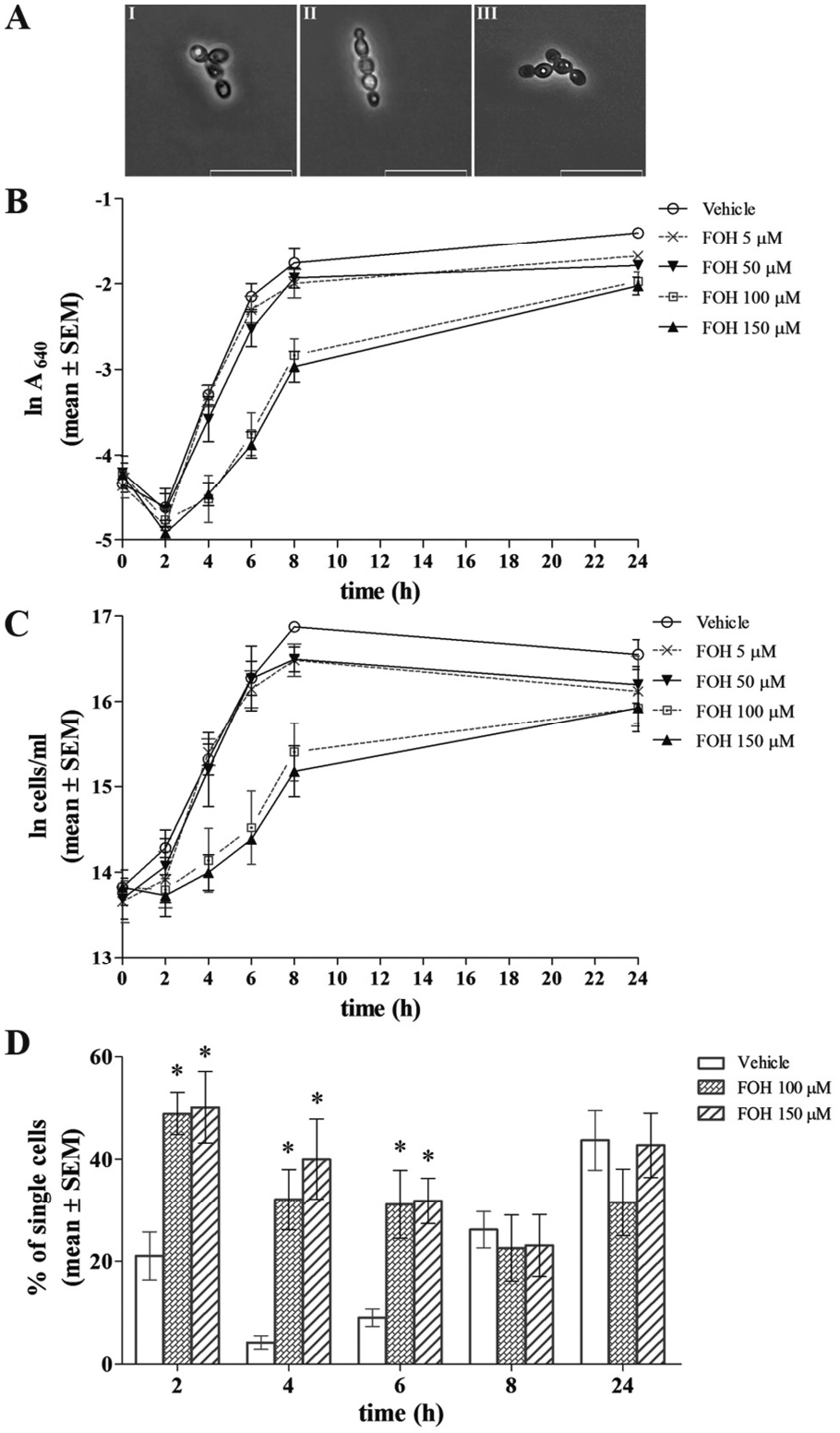

Fig.3.1-3. Characterization of farnesol (FOH) effect in *C. tropicalis*. Morphology (***A***) 2 h after vehicle (***I***), FOH 5 µM (***II***), and FOH 150 µM (***III***) treatments is exemplified (bar = 20 µm). Growth is expressed as mean ln A_{640} ± SEM (***B***) and mean ln cells/ml ± SEM (***C***). Progression through the cell cycle is expressed as mean percentage of single cells ± SEM (***D***). At 0 h, the mean percentage of single cells was 49%. At each time point statistically significant differences (farnesol treatment vs control) are indicated with an asterisk (*, P< 0.05). For simplicity statistical significances were omitted from the panels B and C.

DISCUSSION

The role of farnesol in *C. albicans* and some NCAC species planktonic [3, 9, 12] and biofilm cells [4, 8, 13, 14] has been investigated in the last years. Herein, we aimed to expand previous studies and evaluate the effect of farnesol in other NCAC species planktonic cells, selecting for that one type strain representing *C. glabrata*, *C. krusei*, and *C. tropicalis*.

This study shows that farnesol does not regulate the morphology of the NCAC species evaluated (Fig.3.1-1 *A* to Fig.3.1-3 *A*). This suggests that the role of farnesol in morphology regulation may be specific of *C. albicans* [3] and *C. dubliniensis* [12], probably related to the ability of these *Candida* species to switch between forms in response to environmental factors, which is not a known specific trait of other *Candida* species such as *C. glabrata*, *C. krusei*, and *C. tropicalis* [15].

However, the present results showed that farnesol has a concentration dependent effect in NCAC species growth (Fig.3.1-1 *B* and *C* to Fig.3.1-3 *B* and *C*). At levels considered physiological relevant for *C. albicans* (below 5 μM) [16, 17], farnesol did not affect NCAC species growth (Fig.3.1-1 *B* and *C* to Fig.3.1-3 *B* and *C*). In accordance, the effect of farnesol treatment in different cell types [recently reviewed by Langford *et al.* [18]] show that in general, the compound levels that compromise growth are ≥ 5 μM. In fact, at supraphysiological levels (≥ 50 μM), farnesol impaired NCAC species growth (Fig.3.1-1 *B* and *C* to Fig.3.1-3 *B* and *C*). In light of the recent controversies [18], it should be noted that under similar experimental conditions farnesol up to 150 μM did not affect *C. albicans* and *C. dubliniensis* growth [12], but at concentrations ≥50 μM impaired *C. parapsilosis* growth [9]. Particularly, the minimal farnesol level required to reduce cell growth differed between the different NCAC species: 50 μM for *C. krusei* (Fig.3.1-2 *B* and *C*) and 100 μM for *C. glabrata* (Fig.3.1-1 *B* and *C*) and *C. tropicalis* (Fig.3.1-3 *B* and *C*).

In general, the effects of farnesol on NCAC species levelled off beyond 100 μM (Fig.3.1-1 *B-D* to Fig.3.1-3 *B-C*; Table 3.1-1), suggesting a threshold level for NCAC species farnesol susceptibility. Although it can be argued that the effect induced by higher farnesol levels is non-specific, no differences were observed between farnesol 0 μM and Triton X-100 or methanol

treatments (data not shown). In fact, a similar behaviour has been described for farnesol treatment of the fungus *Penicillium expansum* [19] and of tobacco cells [20]. Additionally, further inspection of the parameters evaluated reveals that in some cases the effects elicited by the different farnesol concentrations are distinct. Specifically, (i); farnesol 50 μM showed a weak detrimental effect in *C. krusei* growth (Fig.3.1-2 *B* and *C*) without changes in cell cycle progression (Fig.3.1-2 *D*), in comparison to the higher farnesol levels; (ii) farnesol 150 μM, but not 100 μM, induced a reduction in *C. glabrata* cell number/ml at the end of 24 h (Fig.3.1-1 *C*); and (iii) farnesol 150 μM, but not 100 μM, reduced the ability of *C. tropicalis* to form colonies (Table 3.1-1).

In addition, this study suggests that the effect of farnesol in cell viability is species dependent (Table 3.1-1). In contrast to *C. glabrata* and *C. krusei*, *C. tropicalis* farnesol mediated growth inhibition (Fig.3.1-3 *B* and *C*) reflect, at least in part, loss of cell culturability and/or cell membrane damage (Table 3.1-1). Similarly, it has been reported that farnesol slightly reduces *C. parapsilosis* cell viability, which was suggested to be associated with the expression of cell aging genes [9]. However, it has been shown that farnesol prevented the normal cell division of *S. cerevisiae* cells for a long period, after which the cells can recover the ability to form colonies [21]. So, it cannot be excluded that the changes in *C. tropicalis* viability (Table 3.1-1) are transient. It was also noted that farnesol 100 μM treated *C. tropicalis* cells exhibited membrane changes but retained the ability to form colonies, in contrast to farnesol 150 μM treated cells (Table 3.1-1). These results suggest that farnesol at 100 μM increases the permeability/fluidity of the membrane, whereas at 150 μM is more cytotoxic, disrupting the cell membrane and thus compromising cell viability. Therefore, the assessment of other parameters from which membrane damage could be inferred, such as, potassium and adenosine triphosphate leakage, membrane potential, and membrane ultrastructure may clarify the impact of farnesol treatment on *C. tropicalis*.

Additionally, the cell cycle progression of farnesol treated cells was species specific (Fig. 3.1-1 *D* to Fig. 3.1-3 *D*). Cell growth and cell cycle are highly coordinated processes. The mechanisms that couple these processes include: (i) the dependency of cell cycle progression on growth and vice versa, (ii) their simultaneous coordination, and (iii) the independent

regulation of both [22]. On one hand, data showed that farnesol 100 and 150 μM triggers a delay in *C. glabrata* S-G2/M cell cycle phase (Fig.3.1-1 *D*), in accordance with a delay in the log growth phase (Fig.3.1-1 *B* and *C*). On the other hand, farnesol 100 and 150 μM induced a delay in *C. krusei* (Fig.3.1-2 *D*) and *C. tropicalis* (Fig.3.1-3 *D*) G0/G1 cell cycle phases, in line with the prolonged lag phase exhibited by these species (Fig.3.1-2 *B* and *C* and Fig.3.1-3 *B* and *C*) in the presence of farnesol. The fact the *C. krusei* and *C. tropicalis* cell size does not seem to be affected by farnesol (Fig.3.1-2 *A* and Fig.3.1-3 *A*), suggests that for these strains the cell cycle delay is a consequence of the growth delay [22]. An additional study has shown that farnesol does not induce cell cycle changes in *C. parapsilosis* exponential growing cells [9]. However, as several lines of evidence point to a correlation between the growth phase of the inocula and the effects elicited by farnesol [23, 24], the challenge of *C. glabrata*, *C. krusei*, and *C. tropicalis* exponentially growing cells with farnesol may clarify if cell cycle changes are inocula growth phase or species specific. Additionally, synchronizing the cell cycle of NCAC species cells before farnesol treatment, or the use of 5-bromodeoxyuridine [25] may be helpful in the determination of cell cycle phase durations and eventually in the discrimination of a subpopulation of farnesol tolerant cells. Finally, the determination of the cycle checkpoints regulated upon farnesol treatment could clarify how farnesol treated cells coordinate cell growth and division, and how that correlates with cell ability to withstand the toxic effects of farnesol recovering from a transient cell cycle arrest.

In summary, this study gives new insights into farnesol as a potential regulator of *C. glabrata*, *C. krusei*, and *C. tropicalis* physiology. The present work shows that farnesol ≥ 50 μM impairs the tested NCAC species cell growth. Although a similar net effect and ability to overcome farnesol toxicity was observed, these data suggest that farnesol has distinct effects in the different NCAC species.

Update

Recent work demonstrated that farnesol inhibits *C. glabrata*, *C. krusei*, and *C. tropicalis* planktonic cells growth [26].

REFERENCES

1. Pfaller, M. A. & Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**, 133-163.
2. Ramage, G., Martinez, J. P., & Lopez-Ribot, J. L. (2006) *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* **6**, 979-986.
3. Hornby, J. M., Jensen, E. C., Lisec, A. D. *et al.* (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982-2992.
4. Ramage, G., Saville, S. P., Wickes, B. L., & Lopez-Ribot, J. L. (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* **68**, 5459-5463.
5. Shirtliff, M. E., Krom, B. P., Meijering, R. A. *et al.* (2009) Farnesol-induced apoptosis in *Candida albicans*. *Antimicrob Agents Chemother* **53**, 2392-2401.
6. Fairn, G. D., Macdonald, K., & McMaster, C. R. (2007) A chemogenomic screen in *Saccharomyces cerevisiae* uncovers a primary role for the mitochondria in farnesol toxicity and its regulation by the Pkc1 pathway. *J Biol Chem* **282**, 4868-4874.
7. Savoldi, M., Malavazi, I., Soriani, F. M. *et al.* (2008) Farnesol induces the transcriptional accumulation of the *Aspergillus nidulans* Apoptosis Inducing Factor (AIF)-like mitochondrial oxidoreductase. *Mol Microbiol* **70**, 44-59.
8. Jabra-Rizk, M. A., Shirtliff, M., James, C., & Meiller, T. (2006) Effect of farnesol on *Candida dubliniensis* biofilm formation and fluconazole resistance. *FEMS Yeast Res* **6**, 1063-1073.
9. Rossignol, T., Logue, M. E., Reynolds, K., Grenon, M., Lowndes, N. F., & Butler, G. (2007) Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob Agents Chemother* **51**, 2304-2312.
10. Coder, D. (1997) Assessment of cell viability. Current Protocols in Cytometry. John Wiley & Sons, Inc., p 9.2.1-9.2.14.
11. Fortuna, M., Sousa, M. J., Corte-Real, M., Leão, C., Salvador, A., & Sansonetty, F. (2000) Cell cycle analysis of yeasts. Current Protocols in Cytometry 11.13.1-11.13.9.
12. Henriques, M., Martins, M., Azeredo, J., & Oliveira, R. (2007) Effect of farnesol on *Candida dubliniensis* morphogenesis. *Lett Appl Microbiol* **44**, 199-205.
13. Laffey, S. F. & Butler, G. (2005) Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151**, 1073-1081.
14. Martins, M., Henriques, M., Azeredo, J., Rocha, S. M., Coimbra, M. A., & Oliveira, R. (2010) *Candida* species extracellular alcohols: production and effect in sessile cells. *J Basic Microbiol* **in press**.
15. Calderone, R. A. (2002) Taxonomy and biology of *Candida*. In: Calderone, R. A. (ed) Candida and Candidiasis. Washington: ASM, pp 15-27.
16. Nickerson, K. W., Atkin, A. L., & Hornby, J. M. (2006) Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl Environ Microbiol* **72**, 3805-3813.

17. Weber, K., Sohr, R., Schulz, B., Fleischhacker, M., & Ruhnke, M. (2008) Secretion of *E,E*-farnesol and biofilm formation in eight different *Candida* species. *Antimicrob Agents Chemother* **52**, 1859-1861.
18. Langford, M. L., Atkin, A. L., & Nickerson, K. W. (2009) Cellular interactions of farnesol, a quorum-sensing molecule produced by *Candida albicans*. *Future Microbiol* **4**, 1353-1362.
19. Liu, P., Luo, L., Guo, J. *et al.* (2010) Farnesol induces apoptosis and oxidative stress in the fungal pathogen *Penicillium expansum*. *Mycologia* **102**, 311-318.
20. Hemmerlin, A. & Bach, T. J. (2000) Farnesol-induced cell death and stimulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in tobacco cv bright yellow-2 cells. *Plant Physiol* **123**, 1257-1268.
21. Machida, K., Tanaka, T., Yano, Y., Otani, S., & Taniguchi, M. (1999) Farnesol-induced growth inhibition in *Saccharomyces cerevisiae* by a cell cycle mechanism. *Microbiology* **145**, 293-299.
22. Neufeld, T. P. & Edgar, B. A. (1998) Connections between growth and the cell cycle. *Curr Opin Cell Biol* **10**, 784-790.
23. Langford, M. L., Hasim, S., Nickerson, K. W., & Atkin, A. L. (2009) Activity and toxicity of farnesol towards *Candida albicans* is dependent on growth conditions. *Antimicrob Agents Chemother* **54**, 940-942.
24. Uppuluri, P., Mekala, S., & Chaffin, W. L. (2007) Farnesol-mediated inhibition of *Candida albicans* yeast growth and rescue by a diacylglycerol analogue. *Yeast* **24**, 681-693.
25. Jayat, C. & Ratinaud, M. H. (1993) Cell cycle analysis by flow cytometry: principles and applications. *Biol Cell* **78**, 15-25.
26. Weber, K., Schulz, B., & Ruhnke, M. (2010) The quorum-sensing molecule *E,E*-farnesol-its variable secretion and its impact on the growth and metabolism of *Candida* species. *Yeast* **in press**.

Chapter 3.2 Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells

Abstract

Morphogenesis control by chemical signaling molecules is beginning to be highlighted in *Candida* biology. The present study focuses on morphogenic compounds produced in situ by *Candida albicans* and *Candida dubliniensis* during planktonic and biofilm growth that may at least partially substantiate the effect promoted by supernatants in morphogenesis. For both species, planktonic vs biofilm supernatants were analyzed by headspace-solid-phase microextraction and gas chromatography-mass spectrometry. Both planktonic cells and biofilm supernatants of *C. albicans* and *C. dubliniensis* contained isoamyl alcohol, 2-phenylethanol (phenylethanol), 1-dodecanol, *E*-nerolidol (nerolidol), and *E*,*E*-farnesol (farnesol). Alcohol secretion profiles were species, culture mode, and growth time specific. The addition of exogenous alcohols to the cultures of both species inhibited the morphological transition from the yeast to the filamentous form by up to 50%. The physiological role of these alcohols was put to evidence by comparing the effects of 96-h cultured supernatants with Cocktail synthetic mixtures containing isoamyl alcohol, phenylethanol, nerolidol, and farnesol at concentrations determined herein. All Cocktail mixtures elicited a morphological effect similar to that observed for the corresponding supernatants when used to treat *C. albicans* and *C. dubliniensis* cultures, except for the effect of the 96-h *C. dubliniensis* planktonic supernatant culture on *C. albicans*. Overall, these results reveal a group of alcohol extracellular signaling molecules that are biologically active with *C. albicans* and *C. dubliniensis* morphogenesis.

The results presented over this chapter were:

(i) published as,

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(iii) presented as poster communication,

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Martins, M., Henriques, M., Azeredo, J., Oliveira, R. Effect of farnesol from *Candida albicans* and *Candida dubliniensis* on *Candida dubliniensis* morphology. 2007. **Biofilms 2007 - 4th ASM Conference on Biofilms**, March 25th – 29th, Quebec, Canada, B188, p. 118-119.

INTRODUCTION

Candida albicans is a major human pathogen, causing life-threatening disseminated infections. Candidiasis epidemiology was recently reviewed [1], emphasizing the increase in invasive *Candida* infections, mainly due to an augment ascribed to Non-*Candida albicans Candida* (NCAC) species. This is supported by the increased use of immunosuppressive therapy and broad-spectrum anti-mycotic prophylaxis [1]. In particular, *Candida dubliniensis*, a NCAC species, colonizes mostly the adult population carrying immunodeficiency virus, causing oral lesions and bloodstream infections [2].

Some mechanisms have been implicated in *C. albicans* and *C. dubliniensis* pathogenicity, namely the morphological switch from the yeast to filamentous form. In this sense, one of the key issues in *C. albicans* and *C. dubliniensis* is the elucidation of the mechanisms involved in morphogenesis control.

Some environmental factors have been reported as determinants of morphological regulation, particularly in *C. albicans*. For instance, hyphal inducing conditions include stimuli such as increase in temperature, pH, serum, nutrient starvation, and cell density [3]. In particular, at high cell densities, *C. albicans* grows in the yeast form, while at low densities filamentation is observed [4]. Cell density regulation is a key process not only in the planktonic mode of growth, but also in biofilms, that are found in many medical devices and constitute a clinically relevant issue [5]. In biofilms, this process may allow overpopulation and nutrient load control, as well as filamentation regulation, important players in sessile development [6]. The morphologic switch mediated by auto-regulatory metabolites produced by *C. albicans* cells has been investigated. Namely, 2-phenylethanol (phenylethanol), tryptophol [7, 8], and farnesoic acid [9] were pointed out to control cellular germination. Moreover, Hornby *et al.* [4] showed that *C. albicans* produces *E*,*E*-farnesol (farnesol) that, when accumulated at a threshold level, inhibits the mycelial growth in a cell density trend. Subsequent studies demonstrated that this molecule, produced in situ by planktonic *C. albicans* cultures, also prevents biofilm formation [10]. The increasing knowledge on cell-cell communication molecules highlighted tyrosol as a quorum sensing signal in *C. albicans* [11]. Regarding morphological control, tyrosol accelerates *C. albicans* germ tube formation in inducing conditions, but does not elicit this effect in non-inducing conditions. Although it is produced by planktonic and biofilm cells [12], its effect on biofilm formation is not clear yet [8, 12].

Nowadays, it is known that among *Candida* species farnesol effects are not restricted to *C. albicans*. Specifically, farnesol prevents yeast to pseudohyphae transition in *C. dubliniensis* [13], and has no effect on *Candida parapsilosis* morphology [14] although it reduces biofilm formation in both NCAC species [15, 16].

In this study, the main goal was to gain insights into *C. albicans* and *C. dubliniensis* morphogenesis control through signaling molecules released into the environment.

Material and Methods

Chemicals

Standard alcohols were all obtained from Sigma. Stock solutions were prepared in methanol (except for isoamyl alcohol), and then used at the following concentrations: isoamyl alcohol, 46 mM, 23 mM and 46 μM; phenylethanol, 500 μM and 5 μM; 1-dodecanol (dodecanol), 200 μM, 2 μM and 2 nM; *E*-nerolidol (nerolidol) and farnesol, 1.5 μM and 1.5 nM. Cocktail mixtures mimicking supernatant fractions from 96-h cultures contained isoamyl alcohol, phenylethanol, nerolidol, and farnesol at the following concentrations: 94 μM, 70 μM, 3.2 nM, 18 nM and 48 μM, 87 μM, 9.5 nM, 5.3 nM (for *C. albicans* planktonic and biofilm forms, respectively); 77 μM, 61 μM, 1.5 nM, 2.0 nM and 53 μM, 93 μM, 5.3 nM, 1.5 nM (for *C. dubliniensis* planktonic and biofilm forms, respectively).

Strains and culture conditions

Two *Candida* species were used in this study: *C. albicans* CECT 1472 (Colección Española de Cultivos Tipo, Spain) and *C. dubliniensis* CBS 7987 (Centraalbureau voor Schimmelcultures, Germany). Prior to each experiment, both *Candida* species were maintained at 37°C in Sabouraud dextrose agar. After that, cells were grown in Sabouraud dextrose broth at 37°C for 24 h on a mechanical shaker at 130 rpm. Subsequently, cells were washed twice with ultrapure water and counted in a hematocytometer. Standardized cell suspensions were prepared at a cell density of 1×10^6 cells/ml except if otherwise stated, in 1× Roswell Park Memorial Institute 1640 (RPMI) medium (Sigma) buffered with 3-[N-morpholino] propanesulfonic acid (Sigma) (final concentration 0.165 M; pH 7.0).

Preparation of culture supernatants

For planktonic supernatant preparation, 120 ml of standardized cell suspensions were inoculated in 300 ml flasks and incubated at 37°C, 130 rpm. At every 24 h of incubation the culture medium was partially renewed (30 ml). At the selected times, supernatant fractions were filter sterilized (0.22 μm) and stored at 4°C.

For biofilm supernatant, standardized cells suspensions (4 ml) were inoculated into six well plates and incubated at 37°C, 130 rpm. After 3 h non-adherent microoganisms were removed and fresh medium was added to allow biofilm formation from 24 to 96 h at 37°C and 130 rpm. After every 24 h of incubation, broth was partly renewed (1 ml). Before storage the supernatant fractions were prepared as above.

Biomass dry weight measurements

At the end of incubation periods, 1 ml of either planktonic or ressupended biofilm cells was filtered through pre-weighted filters (0.45 μm) and washed three times with ultrapure sterilized water. Filters were dried at 60°C until constant weight and cell dry weight was determined. This was repeated at least four times.

Planktonic and biofilm cells characterization

Planktonic cultures were prepared as described above and, at selected times, cellular morphology was observed directly by light microscopy.

Biofilms were formed by seeding 2 ml of the standardized cell suspension in 24 well plates and incubating for 3 h at 37°C, 130 rpm. Then, non-adherent cells were aspirated and fresh medium was added. Plates were incubated at 37°C, 130 rpm. At the selected time points, biofilms were washed three times with ultrapure sterile water. Samples were alcohol dehydrated (70% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 20 min), and air dried for 20 min. Samples were kept in a desiccator until the bottoms of the wells were cut and coated with gold. Biofilm examination was performed on a Leo scanning electron microscope.

Supernatant alcohols analyses

Supernatant alcohols were extracted using headspace-solid-phase microextraction (HS-SPME) and analysed by gas chromatography-mass spectrometry (GC-MS). The SPME holder for manual sampling and fibre used were purchased from Supelco (Aldrich, Bellefonte, PA). The SPME device included a fused silica fibre, partially cross-linked carbowax-divinylbenzene (CW/DVB) with 65 μm film thickness, containing a liquid polymer and solid particles [17]. The CW/DVB coating fibre is recommended for small and polar molecules (molecular weight between 40 and 275), such as the compounds under study. The SPME fibre was conditioned at 250°C for 30 min in the GC injector port, according to the manufacturer's recommendations. For headspace sampling, 20 ml of each supernatant fractions was introduced into a 60 ml glass vial. The vial was capped with a polytetrafluoroethylene septum and an aluminium cap (Chromacol Ltd, Herts, UK) after the addition of 4 g of NaCl and a 20 × 5 mm stirring bar (200 rpm), and was placed in a thermostat bath adjusted to 40 ± 0.1°C for 15 min to transfer the compounds from the sample to the headspace. Following this step, the SPME fibre was manually inserted into the sample vial headspace for 45 min and then introduced into the GC injection port at 250°C and kept for 5 min for alcohol thermal desorption. The GC-MS parameters were established according to Coelho *et al.* [18]. The injection port was lined with a 0.75 mm ID splitless glass liner. The desorbed volatile compounds were separated in a GC-MS Agilent Technologies 6890 N Network gas chromatograph, equipped with a 30 m × 0.32 mm ID, 0.25 μm film thickness DB-FFAP model fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA), connected to an Agilent 5973 quadrupole mass selective detector. Splitless injection mode was used (5 min). The oven temperature was programmed from 35 to 220°C at 2°C/min, and the transfer line was heated at 250°C. Helium carrier gas had a flow of 1.7 ml/min. The mass spectrometer was operated in the electron impact mode at 70 eV scanning the range 33-300 *m/z* in a 1-s cycle, in a full scan acquisition mode. The identification of *Candida* metabolites was achieved comparing the GC retention times and mass spectra, with those of the pure standard compounds. All mass spectra were also compared with the data system library (Wiley 275). A comparable analysis was done with growth medium and no interfering substances were found into or near the retention times of those compounds. The quantification was performed by preparation of RPMI solutions containing pure standards in the same conditions of the samples. For each compound, appropriate concentration ranges were chosen in order to include sample

concentrations. Standard curves were generated for GC-MS peak areas versus concentration of each compound (R^2>0.98), with quantification relying above quantification limits. All measurements were made with, at least, two replicates, each replicate representing the analysis of one different aliquot (20 ml) of each supernatant fraction. This approach allows the GC peak area data to be used as an indirect approach to estimate the relative content of each volatile compound. Blanks, corresponding to the analysis of the coating fibre not subjected to any extraction procedure, were run between sets of three analyses. Alcohols analyses in planktonic and biofilm supernatant fractions were carried out in duplicate for each sample within results agreeing with ≤ 15%.

Filamentation and growth assays

Standardized cell suspensions were prepared in 2 × RPMI and diluted (1:1) with either *C. albicans* or *C. dubliniensis* supernatant prepared as described above. Cultures were incubated for 12 h at 37°C, 130 rpm. A control of RPMI without culture supernatant was used.

Standardized cell suspensions were prepared in RPMI with the specific compounds at specified concentrations or in Cocktail mixtures mimicking supernatant fractions from 96 h culture. All the suspensions were incubated for 12 h, at 37°C, 130 rpm. For each experiment an equal volume of solvent was used as an appropriate control, as well as culture medium without the specific compound. Cell morphology was evaluated according to Henriques *et al.* [13]. All experiments were repeated at least three times with duplicate samples and 10 fields per slide were examined (at least 150 cells). Morphological analyses of methanol treated and untreated controls agreed in more than 97%. Assays regarding supernatant effect were standardized against cell dry weight.

To investigate the effect of isoamyl alcohol, phenylethanol, dodecanol, nerolidol, and farnesol on *Candida* cell growth, batches of cultures were grown for 12 h at 37°C, 130 rpm in the presence or absence of these alcohols as well as appropriate methanol controls, and absorbance was measured at 620 nm, and compared to that of controls. Cell growth monitoring was performed in three independent assays. No growth inhibition was detected in methanol treated controls.

Statistical analyses

Statistical analyses was performed using the GraphPad Prism Software version 5.00 for Windows. Normality of data distribution was tested by the Kolmogorov–Smirnov method. Two tailed paired t-test method was used to determine differences between: (i) means of optical densities of *C. albicans* and *C. dubliniensis* cultures grown in RPMI medium and RPMI plus tested alcohols, (ii) means of the effect of 96 h supernatant vs the corresponding Cocktail mixture on both *Candida* species filamentation inhibition, and (iii) means of RPMI grown cells vs methanol controls, in growth and filamentation assays. Statistical significance of groups' means regarding (i) cultures cell dry weight, (ii) the effect of culture supernatants, and (iii) that of synthetic alcohols vs supernatants in *Candida* species morphology were evaluated using a one-way ANOVA. Subsequent comparisons were performed using Tukey's post-hoc test. Two-way ANOVA was used to test for eventual interactions between: (i) cultures cell dry weight, and (ii) the effect of culture supernatants in cells morphology and *Candida* species.

RESULTS

Characterization of planktonic and biofilm cells

The morphology of *C. albicans* and *C. dubliniensis* planktonic cells, which produced the supernatant, was monitored by light microscopy in 24-, 48-, 72-, and 96-h cultures (data not shown). Most of *C. albicans*, at 24 h, comprised cells in the hyphae form, while from 48- and 72- h an increase in yeast and pseudohyphae was found. Concerning *C. dubliniensis,* 24 h cultures presented a prevalence of pseudohyphae but from 48 to 96 h an increase in yeast forms was observed.

Scanning electron microscopy observation of *C. albicans* biofilms showed cells in the filamentous form, and some yeast cells, despite biofilm age (Fig.3.2-1 *I*). *C. dubliniensis* 24 and 48 h biofilms comprised pseudohyphae and yeast forms (Fig.3.2-1 *II-A* and *II-B*). However, 72 h biofilms were composed mostly of yeast cells and pseudohyphae were rarely observed (Fig.3.2-1 *I-C*). Additionally, 96-h *C. dubliniensis* biofilms (Fig.3.2-1 *IID*) contained again a heterogeneous mixture of yeast and pseudohyphae forms.

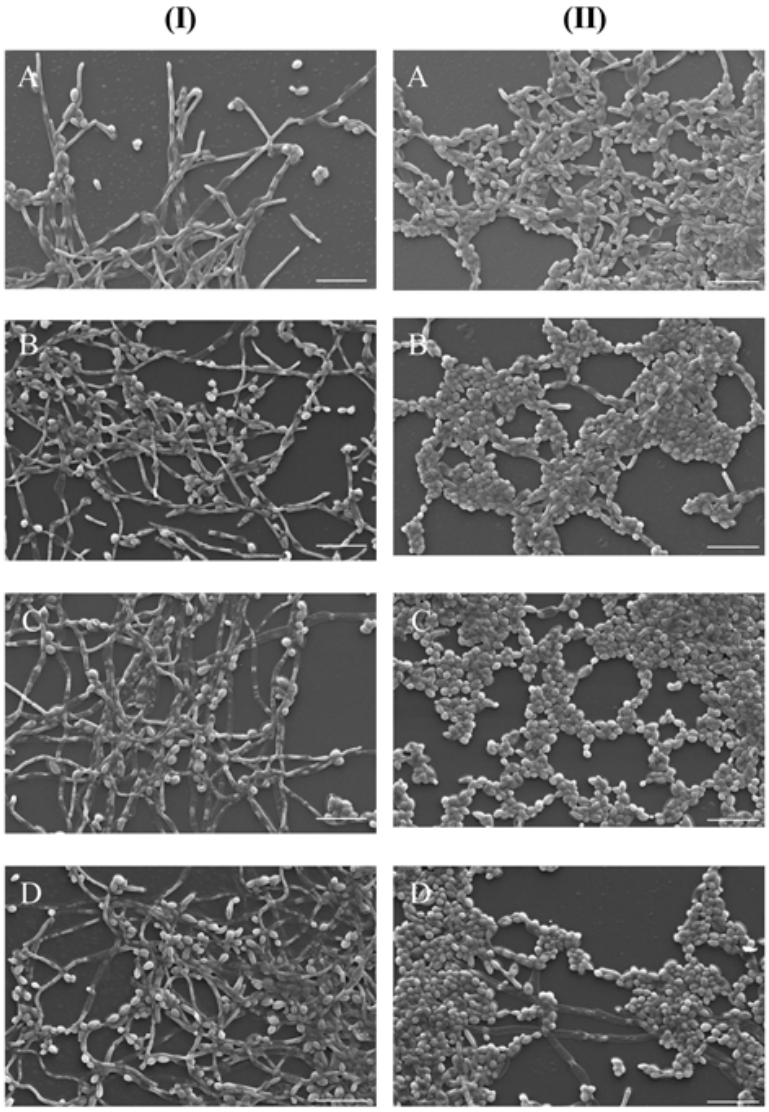

Fig.3.2-1. Scanning electron microscopy of *C. albicans* (***I***) and *C. dubliniensis* (***II***) biofilms grown over 24 h (***A***), 48 h (***B***), 72 h (***C***), and 96 h (***D***). Bar, 20.0 μm.

Effect of planktonic and biofilm culture supernatants on *C. albicans* and *C. dubliniensis* filamentation

One of the aims of this work was to assess the effect of *C. albicans* and *C. dubliniensis* planktonic and biofilm supernatant on inter and intra species morphological transition. It should be noted that for each assay the percentage of inhibition reveals the relation between the percentage of hyphae inhibition, in *C. albicans*, and the percentage of pseudohyphae inhibition, in *C. dubliniensis*. Also the averages of biomass dry weight from *C. albicans* planktonic cells were significantly higher than biofilm ones, except at 24 h. An opposite relation was observed

for *C. dubliniensis* cultures. Comparing both species cell dry weight no statistical differences were detected, except for 24 h biofilms.

C. albicans supernatants displayed an inhibitory activity regarding morphological transition from yeast to filamentous form, on both species (Fig.3.2-2 ***I***). For *C. albicans* cells treated with its own planktonic supernatant (Fig.3.2-2 *I-A*) percentages of inhibition of hyphae formation significantly increased from 48 to 96 h. The evaluation of the effect of the same supernatants in *C. dubliniensis* (Fig.3.2-2 *I-B*) showed the highest inhibition of pseudohyphae formation for the 96 h fraction ($P<0.001$). *C. albicans* biofilm supernatant, markedly increased the percentage of inhibition with increasing culture time in both *Candida* species (Fig.3.2-2 *I-A* and *I-B*).

Furthermore, the use of *C. dubliniensis* supernatant, from either planktonic or biofilm

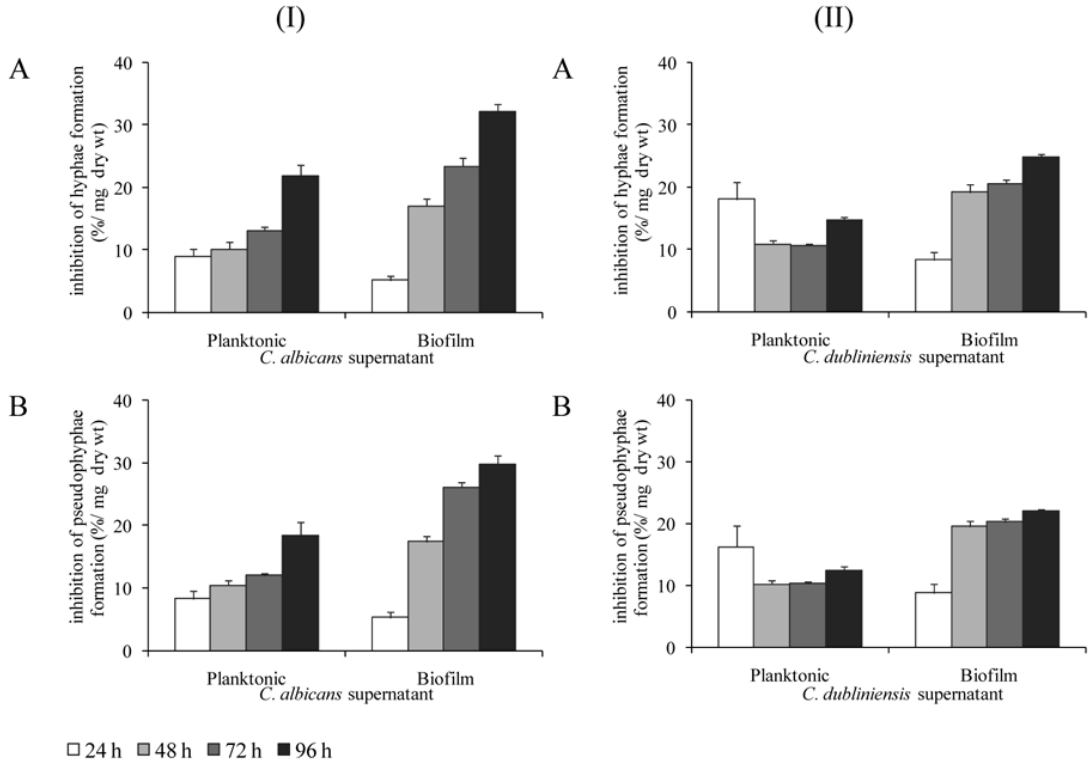

Fig.3.2-2. Effect of time on *C. albicans* supernatants (***I***) and *C. dubliniensis* supernatants (***II***) from planktonic and biofilm cultures on the morphology of 12-h planktonic growth of *C. albicans* (***A***) and *C. dubliniensis* (***B***). Filamentation was assessed as the number of hyphae in *C. albicans* and number of pseudohyphae in *C. dubliniensis* per 10 slides counted in duplicate samples. Control cells were incubated in RPMI medium, representing >90% of hyphae plus 2% of pseudohyphae for *C. albicans* and >60% of pseudohyphae for *C. dubliniensis*. The percentages of inhibition in the three independent assays were determined as a mean percentage of reduction (vs control), as a function of mg of dry weight ± standard deviation (SD).

cells, also prevented the yeast to filamentous form transition not only in *C. albicans* but in *C. dubliniensis* as well (Fig.3.2.2 *II*). Regarding planktonic supernatant effect in the morphological transition of *C. albicans* (Fig.3.2-2 *II-A*) and *C. dubliniensis* (Fig.3.2-2 *II-B*) cells, the highest effect was observed for the 24 h fraction ($P<0.001$). Additionally, the 24 h biofilm supernatant of *C. dubliniensis* had significantly lower effect, in both *Candida* species (Fig.3.2-2 *II*).

Both *C. albicans* (Fig.3.2-2 *I*) and *C. dubliniensis* (Fig.3.2-2 *II*) biofilm spent media from older biofilms (72 and 96 h) were significantly more effective, in the reduction of filamentation than their planktonic equivalents in both species.

Overall, the present results show that supernatant composition may be dependent on *Candida* species, differing not only due to the mode of growth, but also along time.

Characterization of planktonic and biofilm supernatant composition

Supernatant samples were analysed by HS-SPME GC-MS. Comparison of retention times, and mass spectra with library, and chemically synthesized standards ascertained five of the peaks as isoamyl alcohol, phenylethanol, dodecanol, nerolidol, and farnesol (data not shown). The results expressed as a function of cell dry weight (Table 3.2-1) reveal that these compounds are continuously excreted by planktonic and biofilm cells.

Regarding the amounts of isoamyl alcohol in *C. albicans* planktonic supernatants, they were very similar (Table 3.2-1). However, 24 h biofilms released less isoamyl alcohol than older ones. For *C. dubliniensis* planktonic cells (Table 3.2-1) the highest amount of isoamyl alcohol was observed for 24 cultures, while in biofilms supernatant isoamyl alcohol concentration slightly increased with maturation.

Considering phenylethanol release by *C. albicans* planktonic and biofilm cells (Table 3.2-1), this aromatic alcohol increased into supernatant medium, during the transition from 24 to 96h of growth time. In contrast, *C. dubliniensis* biofilm and planktonic cells exhibited different secretion alcohol patterns (Table 3.2-1). Indeed, culture media from 96 h biofilms contained higher phenylethanol levels than the ones obtained from 24 h biofilms, in contrast to an approximately constant level of phenylethanol production exhibited by planktonic cultures supernatant.

Table 3.2-1. Concentration of secreted alcohols in *C. albicans* and *C. dubliniensis* planktonic and biofilm supernatant fractions[a]

Compound		Planktonic culture				Biofilm culture			
		Culture time (h)				Culture time (h)			
		24	48	72	96	24	48	72	96
C. albicans	Isoamyl alcohol (µmol/g cell dry weight)	64.09	58.23	59.36	57.91	12.94	45.17	72.49	46.12
	Phenylethanol (µmol/g cell dry weight)	7.37	18.44	24.62	43.22	5.29	29.39	37.12	88.77
	Nerolidol (nmol/g cell dry weight)	0.70	2.42	2.03	1.96	0.46	2.94	7.53	8.80
	Farnesol (nmol/g cell dry weight)	0.02	0.59	0.25	11.08	0.02	0.16	0.50	5.08
C. dubliniensis	Isoamyl alcohol (µmol/g cell dry weight)	68.79	42.15	20.00	32.90	15.32	25.90	22.70	37.99
	Phenylethanol (µmol/g cell dry weight)	15.40	14.35	19.57	25.80	7.26	41.87	31.46	67.13
	Nerolidol (nmol/g cell dry weight)	0.74	2.29	0.79	0.64	1.30	3.07	4.09	3.80
	Farnesol (nmol/g cell dry weight)	0.07	2.13	0.20	0.84	0.15	1.59	0.65	1.05

[a] Alcohol secretion analyses of planktonic and biofilm supernatant fractions per cell dry weight were carried out in duplicate for each sample, with an agreement of results within ≤15%. For both planktonic and biofilm cultures, at every 24 h of incubation, the medium was partially renewed, as described in Material and Methods.

Nerolidol amounts in *C. albicans* planktonic supernatants (Table 3.2-1) were approximately constant from 48 to 96 h. However, in *C. albicans* biofilm supernatants, the concentration of this sesquiterpenoid continuously raised along with biofilm development, levelling off at 72 h. *C. dubliniensis* displayed a different nerolidol excretion profile according to

the growth mode (Table 3.2-1). In fact, planktonic cells that excreted higher levels of this alcohol belong to the 48-h population. In biofilms, the highest amounts of nerolidol were detected in samples recovered at more than 48 h of growth. Concerning farnesol excretion by *C. albicans*, a similar profile was observed for both planktonic and sessile cells (Table 3.2-1), attaining the highest level at 96 h of growth. In the case of *C. dubliniensis*, farnesol reached the maximum experimental concentration in 48 h supernatant fractions.

Regarding dodecanol, it was detected in all supernatant samples from both *Candida* species at concentrations lower than 2.79 nM (data not shown).

Effect of secreted alcohols on *C. albicans* and *C. dubliniensis* filamentation and growth

The addition of 46 mM of isoamyl alcohol to both *Candida* species growth medium inhibited cell growth (Table 3.2-2). However, for 46 μM and 23 mM of this compound filamentation was inhibited. The increase of the isoamyl alcohol concentration significantly repressed filamentation on both *Candida* species (Table 3.2-2).

In the present study two phenylethanol concentrations were assayed, 5 and 500 μM, and no arrest of cell growth was observed for both concentrations. At 5 μM a reduction in hyphae formation was observed for *C. albicans*, 71.4 ± 15.8%, with 72.9± 17.0% pseudohyphae inhibition for *C. dubliniensis*.

Additionally, dodecanol was assayed at 2 nM, 2 μM, and 200 μM. *C. albicans* revealed no growth inhibition at any of the assayed concentrations. In contrast, 200 μM dodecanol reduced *C. dubliniensis* cell growth in more than 90% (Table 3.2-2). However, dodecanol elicits morphogenic inhibition in both *Candida* species even at a concentration of 2 nM.

In the present situation, 1.5 nM or 1.5 μM of nerolidol promoted a reduction in both *Candida* species filamentation (Table 3.2-2) without growth inhibition. It may be stressed that for both *Candida* species no significant differences were observed concerning the effect triggered by nerolidol at 1.5 nM and 1.5 μM.

Herein, 1.5 μM farnesol control was included and the percentages of inhibition obtained were similar to those previously described [13]. Additionally, 1.5 nM farnesol was noticed to

Table 3.2-2. Effect of secreted alcohols on the growth and filamentation inhibition of planktonic *C. albicans* and *C. dubliniensis*[a]

Compound	Concentration	*C. albicans*		*C. dubliniensis*	
		% growth inhibition	% hyphae inhibition	% growth inhibition	% pseudohyphae inhibition
Isoamyl alcohol	46 mM	77.7 ± 1.4	NA	89.2 ± 0.5	NA
	23 mM	-	95.8 ± 3.0	-	92.4 ± 2.1
	46 µM	-	68.4 ± 12.3	-	70.7 ± 15.6
Phenylethanol	500 µM	-	95.3 ± 1.23	-	91.5 ± 10.7
	5 µM	-	71.4 ± 15.7	-	72.9 ± 17.0
Dodecanol	200 µM	-	98.7 ± 7.9	93.3 ± 0.8	NA
	2 µM	-	69.9 ± 16.8	-	74.5 ± 9.6
	2 nM	-	57.8 ± 14.3	-	54.1 ± 12.1
Nerolidol	1.5 µM	-	64.8 ± 4.7	-	64.6 ± 7.8
	1.5 nM	-	51.1 ± 9.3	-	68.3 ± 5.0
Farnesol	1.5 µM	-	67.5 ± 10.1	-	74.1 ± 17.8
	1.5 nM	-	63.5 ± 10.8	-	57.8 ± 4.8

[a]Percentage of growth inhibition was determined as a reduction of optical density at 620 nm of three independent assays compared to that of controls. Mean optical densities for the controls were 0.51 for *C. albicans* and 0.57 for *C. dubliniensis*. Filamentation was assessed as the number of hyphae in *C. albicans* and the number of pseudohyphae in *C. dubliniensis* per 10 slides, counted in duplicate samples. Control cells were incubated in RPMI medium, presenting >90% of hyphae plus 2% of pseudohyphae for *C. albicans* and >60% of pseudohyphae for *C. dubliniensis*. Percentages of inhibition in the three independent assays were determined as mean percentages of reduction (vs control) ± SD. NA, not applicable.

inhibit cellular germination in both *Candida* species (Table 3.2-2). Similarly to nerolidol, a decrease in farnesol concentration, from 1.5 µM to 1.5 nM, did not result in a significant reduction in filamentation inhibition for *C. dubliniensis*.

On the whole, the results show that these compounds also present in supernatant fractions (Table 3.2-1), regulate yeast cell morphology not resulting in significant growth constraints.

Effect of Cocktail solutions simulating planktonic and biofilm culture supernatants on *C. albicans* and *C. dubliniensis* filamentation

Considering all the conditions assayed (Fig.3.2-3 *I* and *II*), no significant differences were encountered between the effect of supernatant and Cocktail mixtures on *Candida* filamentation inhibition, except for *C. dubliniensis* planktonic supernatant on *C. albicans* (Fig.3.2-3 *I*, condition *3*) (P=0.02).

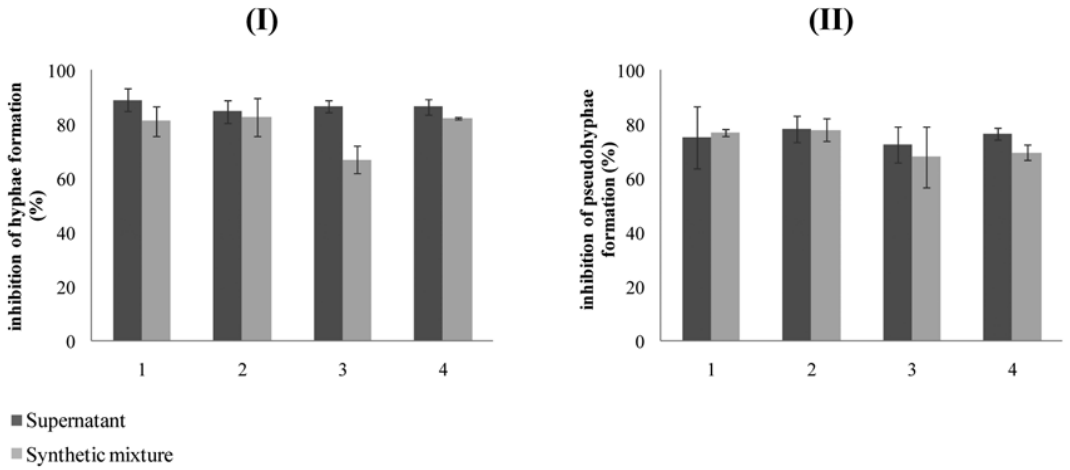

Fig.3.2-3. Effect of 96-h supernatants and corresponding Cocktail mixtures on the morphology of 12-h planktonic cells of *C. albicans* (***I***) and *C. dubliniensis* (***II***). ***Condition 1***, *C. albicans* planktonic supernatant; ***2***, *C. albicans* biofilm supernatant; ***3***, *C. dubliniensis* planktonic supernatant, and ***4***, *C. dubliniensis* biofilm supernatant. Filamentation was assessed as the number of hyphae in *C. albicans* and number of pseudohyphae in *C. dubliniensis* per 10 slides counted in duplicate samples. Control cells were incubated in RPMI medium, representing >90% of hyphae plus 2% of pseudohyphae for *C. albicans* and >60% of pseudohyphae for *C. dubliniensis*. The percentages of inhibition in the three independent assays were determined as the means of percentage of reduction (vs control) ± standard deviations.

DISCUSSION

Within the predominant human pathogenic fungi are the well recognized *C. albicans* and the emerging *C. dubliniensis*. Both *Candida* species are phenotypically very similar and a unifying feature is that both are polymorphic, undergoing interconversion into filamentous and yeast forms. However, *C. dubliniensis* appears to have less ability to cause disease and to ascertain this issue "will enhance our understanding of candidal pathogenesis in general" [2].

Results show that the addition of supernatant medium from planktonic and biofilm cultures prevents filamentation of planktonic cells of the same species (Fig.3.2-2 *I-A* and *II-B*). Besides, similar results were observed crossing supernatant fractions between species (Fig.3.2-2 *I-B* and *II-A*). These data are in accordance with previous reports [16], showing inter- and intra-species effect of *C. albicans* and *C. dubliniensis* supernatant, although using different strains.

Isoamyl alcohol, phenylethanol, dodecanol, nerolidol, and farnesol were detected in supernatant of planktonic and biofilm forms of both *Candida* species by HS-SPME GC-MS. Different studies have reported the effect of these alcohols in filamentation of *Saccharomyces*

cerevisiae and *C. albicans* [4, 8, 19-21]. Previous reports also showed that *C. albicans* yeast cells secret tyrosol and tryptophol at concentrations within micromolar levels [8, 12]. However, those compounds were not detected in the present study. Failure to detect tyrosol and tryptophol may have been due to the SPME experimental conditions used and/or the high solubility of these compounds in water.

Farnesol concentrations determined in the supernatants (Table 3.2-1) were lower than 11.08 nM. Hornby and Nickerson [22] reported that six out of seven *C. albicans* reference strains and clinical isolates produce farnesol at hundreds nanomolar. The exception was the reference strain *C. albicans* ATCC 10231, for which, farnesol, if present, was below the limit of detection of the method used (22.5 nmol/g of cell dry weight). However, using HS-SPME/GC-MS, the limit of detection obtained for farnesol was 0.01 nM (data not shown). Therefore, it seems likely that *C. albicans* ATCC 10231 could produce farnesol within the levels observed for *C. albicans* CECT 1472 and *C. dubliniensis* CBS 7987, used herein.

A time based comparison of supernatant composition (Table 3.2-1) highlights the differential excretion patterns of these alcohols, as previously inferred from supernatant effect in *Candida* species planktonic morphology assays reported in Fig. 3.2-2.

Concerning *C. dubliniensis*, neither of the compounds quantified (Table 3.2-1) supports the high effect triggered by its 24 h planktonic supernatant in the inhibition of self-filamentation (Fig.3.2-2 *B*). Probably, other molecules, not identified in this analysis, may contribute to the observed result.

Moreover, from 48 h onwards the alcohols concentration in *C. albicans* supernatant (planktonic and biofilm) are higher than in *C. dubliniensis* ones (Table 3.2-1). These results suggest that during the ongoing of growth, *C. dubliniensis* can be overcome by *C. albicans*, which corroborates one of the hypotheses postulated by Sullivan *et al*. [2], whereby *C. albicans* would be more “robust” than *C. dubliniensis*. In fact, previous reports concerning *C. albicans* and *C. dubliniensis* fit these results. Reports of *in vitro* studies showed that *C. albicans* has growth competitive advantages over *C. dubliniensis*, more evident at 96 h for planktonic than in biofilm growing conditions [23]. Results presented in Fig.3.2-3 may support this behavior since it can be inferred that *C. dubliniensis* secretes into the environment substances that specifically regulate *C. albicans* morphogenesis. Conversely, in vivo studies [24] showed that whe

inoculated togheter, although the viability of both species was similar in the initial days, *C. dubliniensis* was not detectable at days 8 and 10, post-infection.

Other competitive interactions described in literature show that phenylethanol selectively inhibits certain Gram positive and Gram negative bacteria [25], which could provide growth advantages to *C. albicans* and *C. dubliniensis*.

Another important aspect to be retained is that a distinct biofilm vs planktonic alcohol secretion pattern was found, which is consistent with microbiological regulation of a multicellular behavior that includes protection from environment, nutrient availability and metabolic cooperation. Results from Table 3.2-1 reveal that supernatants from biofilm cells at the latest growth states contain more phenylethanol and nerolidol than the planktonic ones. Interestingly, a similar relation has been described for prostaglandins [26] and tyrosol secretion by *C. albicans* [12]. However, this behavior was not observed for isoamyl alcohol and farnesol. This may at least, in part, explain the higher percentage of inhibition of filamentation induced by biofilm supernatant, in contrast to the planktonic ones (Fig.3.2-2).

Regarding *C. albicans*, an earlier report described that a mixture of nerolidol isomers was two times less active than farnesol [4]. However, the results obtained in this work show that both nerolidol and farnesol have an equivalent activity on cell morphology (Table 3.2-2).

Herein, 5 µM of phenylethanol and 2 nM of dodecanol inhibited (> 50%) both *Candida* species morphological transition from yeast to filamentous form (Table 3.2-2). Other authors described morphological changes only at higher concentrations, 500 µM for phenylethanol [8] and 10 µM for dodecanol [21]. These discrepancies may be due to the distinct design of filamentation assays, although different strain sensitivity to these alcohols may not be excluded.

Furthermore, isoamyl alcohol should be put in parallel with phenylethanol and tryptophol, as inhibitors of *C. albicans* and *C. dubliniensis* filamentous growth but stimulate *S. cerevisiae* filamentation [8, 27]. This differential response may be supported by the fact that *S. cerevisae* response to isoamyl alcohol involves the phosphorylation of Cdc28 at tyrosine 19 by Swe1 [28], a protein not involved in *C. albicans* filamentation [29].

Data in Table 2 show that these alcohols elicit a morphogenic inhibition at physiological concentrations in both *Candida* species. When 96-h supernatant from both species were mimicked in isoamyl alcohol, phenylethanol, nerolidol, and farnesol composition, similar

percentages of inhibition were achieved (Fig.3.2-3). These results suggest that the major morphogenic compounds produced in situ by *C. albicans* and *C. dubliniensis* into the extracellular medium have been identified. The exception was the mixture that mimicked *C. dubliniensis* planktonic supernatant, which elicited a lower morphological response in *C. albicans* than the supernatant (Fig.3.2-3 *I*, condition *3*), suggesting that *C. dubliniensis* may release non-identified compound(s) that specifically control *C. albicans* morphogenesis.

Chen and Fink [8] suggested that in *S. cerevisae* phenylethanol and tryptophol might have some synergic effect regarding invasive growth. However, the effect triggered by the specific mixtures of alcohols (Fig.3.2-3) is slightly lower than the expected from the percentages of inhibition obtained for each single compound (Table 3.2-2). These observations suggest two hypotheses: a competition phenomenon or a saturation process.

Furthermore, it should be stressed that these studies were performed in vitro. Environmental conditions might influence the properties of microorganisms and in the laboratory it is difficult to mimic natural conditions and their heterogeneous parameters [30, 31]. In an attempt to approximate experimental conditions to the natural environment, RPMI medium was used. This may in part simulate human host conditions, however, the amino acid composition of this growth medium might determine the molecules detected (Table 3.2-1), namely isoamyl alcohol and phenylethanol. In addition, cell culture supernatants medium was partially replaced every 24 h, and in fact, in the environment, partial nutrient replacement may occur. Nevertheless, metabolites detected at each time point assayed (Table 3.2-1) may also account to additional metabolism, but this experimental parameter was maintained along all culture times. However, in vivo, the effect and excretion patterns may certainly differ. Some authors noted that farnesol concentrations needed to block germination in *C. albicans* [32] and *C. dubliniensis* [13] in serum are different from the observed in RPMI. So, in vivo, the effectiveness of these alcohols in the control of morphogenesis may be distinct. Moreover, farnesol is not produced under anoxia [33], which is important for some tissue invasion. Thus, it is reasonable to ask if the regulation of synthesis and/or secretion of the other alcohols identified within this study could be different in vivo.

In summary, several lines of evidence provide new insights in *C. albicans* and *C. dubliniensis* metabolites, as well as their role in morphogenesis control. Specifically, isoamyl alcohol, phenylethanol, nerolidol, and farnesol were identified in the extracellular medium of

planktonic and biofilm cells. Besides, these alcohols were shown to inhibit the morphological transition in these *Candida* species, in a similar degree, at physiological concentrations. Despite cell morphology, signalling molecules were secreted at different profiles depending on *Candida* species, stage, and mode of growth (planktonic and biofilm). Overall, this represents a breakthrough in morphogenesis control in both *Candida* species and also inter and intra species interactions may now be better understood.

REFERENCES

1. Pfaller, M. A. & Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**, 133-163.
2. Sullivan, D. J., Moran, G. P., & Coleman, D. C. (2005) *Candida dubliniensis*: Ten years on. *FEMS Microbiol Lett* **253**, 9-17.
3. Ernst, J. F. (2000) Transcription factors in *Candida albicans* - environmental control of morphogenesis. *Microbiology* **146**, 1763-1774.
4. Hornby, J. M., Jensen, E. C., Lisec, A. D. *et al.* (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982-2992.
5. Donlan, R. M. (2001) Biofilm formation: a clinically relevant microbiological process. *Clin Infect Dis* **33**, 1387-1392.
6. Ramage, G., Saville, S. P., Thomas, D. P., & Lopez-Ribot, J. L. (2005) *Candida* biofilms: an update. *Eukaryot Cell* **4**, 633-638.
7. Lingappa, B. T., Prasad, M., Lingappa, Y., Hunt, D. F., & Biemann, K. (1969) Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans*. *Science* **163**, 192-194.
8. Chen, H. & Fink, G. R. (2006) Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev* **20**, 1150-1161.
9. Oh, K. B., Miyazawa, H., Naito, T., & Matsuoka, H. (2001) Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc Natl Acad Sci USA* **98**, 4664-4668.
10. Ramage, G., Saville, S. P., Wickes, B. L., & Lopez-Ribot, J. L. (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* **68**, 5459-5463.
11. Chen, H., Fujita, M., Feng, Q., Clardy, J., & Fink, G. R. (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc Natl Acad Sci USA* **101**, 5048-5052.
12. Alem, M. A., Oteef, M. D., Flowers, T. H., & Douglas, L. J. (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell* **5**, 1770-1779.
13. Henriques, M., Martins, M., Azeredo, J., & Oliveira, R. (2007) Effect of farnesol on *Candida dubliniensis* morphogenesis. *Lett Appl Microbiol* **44**, 199-205.

14. Rossignol, T., Logue, M. E., Reynolds, K., Grenon, M., Lowndes, N. F., & Butler, G. (2007) Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob Agents Chemother* **51**, 2304-2312.
15. Laffey, S. F. & Butler, G. (2005) Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151**, 1073-1081.
16. Jabra-Rizk, M. A., Shirtliff, M., James, C., & Meiller, T. (2006) Effect of farnesol on *Candida dubliniensis* biofilm formation and fluconazole resistance. *FEMS Yeast Res* **6**, 1063-1073.
17. Pillonel, L., Bosset, J. O., & Tabacchi, R. (2002) Rapid preconcentration and enrichment techniques for the analysis of food volatile. A Review. *Lebensm Wiss Technol* **35**, 1-14.
18. Coelho, E., Rocha, S. M., Delgadillo, I., & Coimbra, M. A. (2006) Headspace-SPME applied to varietal volatile components evolution during *Vitis vinifera* L. cv. 'Baga' ripening. *Anal Chim Acta* **563**, 204-214.
19. Dickinson, J. R., Lanterman, M. M., Danner, D. J. *et al.* (1997) A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 26871-26878.
20. Dickinson, J. R., Salgado, L. E., & Hewlins, M. J. E. (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J Biol Chem* **278**, 8028-8034.
21. Hogan, D. A., Vik, A., & Kolter, R. (2004) A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol* **54**, 1212-1223.
22. Hornby, J. M. & Nickerson, K. W. (2004) Enhanced production of farnesol by *Candida albicans* treated with four azoles. *Antimicrob Agents Chemother* **48**, 2305-2307.
23. Kirkpatrick, W. R., Lopez-Ribot, J. L., Mcatee, R. K., & Patterson, T. F. (2000) Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *J Clin Microbiol* **38**, 902-904.
24. Stokes, C., Moran, G. P., Spiering, M. J., Cole, G. T., Coleman, D. C., & Sullivan, D. J. (2007) Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet Biol* **44**, 920-931.
25. Fraud, S., Rees, E. L., Mahenthiralingam, E., Russell, A. D., & Maillard, J. Y. (2003) Aromatic alcohols and their effect on Gram-negative bacteria, cocci and mycobacteria. *J Antimicrob Chemother* **51**, 1435-1436.
26. Alem, M. A. S. & Douglas, L. J. (2005) Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* **54**, 1001-1005.
27. Dickinson, J. R. (1996) 'Fusel' alcohols induce hyphal-like extensions and pseudohyphal formation in yeasts. *Microbiology* **142**, 1391-1397.
28. Martinez-Anaya, C., Dickinson, J. R., & Sudbery, P. E. (2003) In yeast, the pseudohyphal phenotype induced by isoamyl alcohol results from the operation of the morphogenesis checkpoint. *J Cell Sci* **116**, 3423-3431.
29. Wightman, R., Bates, S., Amornrrattanapan, P., & Sudbery, P. (2004) In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization. *J Cell Biol* **164**, 581-591.

30. Palková, Z. (2004) Multicellular microorganisms: laboratory versus nature. *EMBO Rep* **5**, 470-476.
31. Ritz, K. & Crawford, J. W. (1999) Colony development in nutritionally heterogenous environments. In: Gow, N. A. R., Robson, G. D., & Gadd, G. M. (eds) *The fungal colony*. British Mycological Society, pp 49-74.
32. Mosel, D. D., Dumitru, R., Hornby, J. M., Atkin, A. L., & Nickerson, K. W. (2005) Farnesol concentrations required to block germ tube formation in *Candida albicans* in the presence and absence of serum. *Appl Environ Microbiol* **71**, 4938-4940.
33. Dumitru, R., Hornby, J. M., & Nickerson, K. W. (2004) Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. *Antimicrob Agents Chemother* **48**, 2350-2354.

Chapter 3.3 *Candida* species extracellular alcohols: production and effect in sessile cells

ABSTRACT

Cell-cell signaling alcohol molecules were recently identified in *Candida albicans* and *Candida dubliniensis* supernatants. To date, it is not known whether these molecules are produced by other *Candida* species and their role in biofilm formation is not fully clarified. Herein, *Candida parapsilosis* and *Candida tropicalis* extracellular alcohols production by planktonic cultures was analyzed by headspace-solid-phase microextraction and gas chromatography-mass spectrometry. Both *Candida* species extracellular media contained *E,E*-farnesol (farnesol), 1-dodecanol (dodecanol), 2-phenylethanol (phenylethanol), and isoamyl alcohol but not *E*-nerolidol (nerolidol), as produced by *C. albicans* and *C. dubliniensis*. Moreover, the ability of these compounds to regulate *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* sessile cells was assessed by adding the alcohols after 3 h of adhesion and 48 h of biofilm formation. After 24 h, biofilms were analyzed in terms of cellular mitochondrial activity and total biomass. Farnesol affected *C. albicans* and *C. dubliniensis*. Nerolidol and dodecanol elicited *C. parapsilosis* and *C. tropicalis* changes in further biofilm development. *C. tropicalis* was affected by phenylethanol and isoamyl alcohol triggered changes in *C. albicans*, *C. dubliniensis*, and *C. tropicalis* sessile cells. The results demonstrate that almost all of these alcohols are produced by these *Candida* species and also evidence the complexity of biofilm formation.

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Introduction

Candida albicans is the predominant etiological agent of candidiasis, nevertheless, Non-*C. albicans Candida* (NCAC) species have been emerging as significant pathogens [1]. Specifically, *Candida dubliniensis* has been associated with oral candidiasis infections in human immunodeficiency virus infected and acquired immunodeficiency syndrome populations [2]. *Candida parapsilosis* and *Candida tropicalis* are pathogens found in association with candidemia, and are frequently isolated from implanted devices [3, 4].

The success of *Candida* species as pathogens is attributed to survival strategies such as their ability to form biofilms. In fact, biofilms may initiate or prolong infections by providing a source of cells that can colonize local and new sites, and be recalcitrant to antifungal therapy and host immune defenses [3]. *C. albicans* biofilm formation is modulated by the extracellular alcohol *E,E*-farnesol (farnesol) [5]. However, other extracellular alcohols have been identified in *C. albicans* and *C. dubliniensis* supernatants namely *E*-nerolidol (nerolidol), 1-dodecanol (dodecanol), 2-phenylethanol (phenylethanol), and isoamyl alcohol. These molecules were shown to inhibit *C. albicans* and *C. dubliniensis* yeast to filament formation in filamentation inducing conditions [6], but the production and role of these molecules in other *Candida* species has been poorly studied.

This work aimed at investigating the production of extracellular alcohols by *C. parapsilosis* and *C. tropicalis*, and to evaluate the effect of the addition of such alcohols' commercial formulations on *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* biofilm development.

Material and Methods

Chemicals

Farnesol (purity ≥95%), phenylethanol (purity ≥99%), and isoamyl alcohol (purity ≥99%) were obtained from Sigma Aldrich, and nerolidol (purity ≥85%) and dodecanol (purity ≥99.5%) were obtained from Fluka. Pure compounds were stored according to suppliers' instructions.

Isoamyl alcohol working solutions were prepared by direct dilution in growth medium. All the other alcohols stock solutions were prepared in methanol (VWR) and appropriated dilutions

made in growth medium. Stock solutions were prepared freshly before each experiment and appropriated controls were performed in order to ensure compounds stability through the course of the experiments.

Farnesol was already described to regulate biofilm formation of *C. albicans* [5], *C. parapsilosis* [7], and *C. dubliniensis* [8]. In this sense, 150 μM farnesol was used as a control in this study. The selected concentrations of all the other alcohols were based on previously reported physiological and supraphysiological concentrations in *C. albicans* and *C. dubliniensis* [6]. Specifically, they were used at final concentrations of: 1.5×10^{-3}, 1.5, and 150 μM for nerolidol; 2×10^{-3} and 2 μM for dodecanol; 5 and 500 μM for phenylethanol, 46×10^{-3} and 23 mM for isoamyl alcohol.

Strains

In this study the following *Candida* type strains were used: *C. albicans* CECT 1472, *C. dubliniensis* CBS 7987, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750.

Growth conditions

Candida species were stored at -80°C and propagated by streaking a loopfull of cells onto Sabouraud dextrose agar medium (Liofilchem) and incubating at 37°C for 24 h. These stocks were stored at 4°C for no longer than two weeks.

For all experiments, batches of Sabouraud dextrose broth (Liofilchem) (20 ml in 50 ml flasks) were inoculated with freshly grown *Candida* cells, and incubated at 37°C with agitation (130 rpm). After 24 h of growth the cells were harvested by centrifugation, washed twice with ultrapure sterile water, and enumerated in a hematocytometer. Before use in further experiments, 1×10^6 cells/ml standardized cell suspensions were prepared in Roswell Park Memorial Institute 1640 (RPMI) (Sigma), and buffered with 3-[N-morpholino] propanesulfonic acid (Sigma) (0.165 M) to pH 7.0.

Preparation of supernatant samples

C. parapsilosis and *C. tropicalis* culture supernatants were prepared by inoculation of 120 ml of a standardized cell suspension in 300 ml flasks, and incubated at 37°C with shaking at 130

rpm for 24 h. Cell free fractions were recovered by centrifugation and filter sterilized (0.22 µm pore size).

Culture biomass dry weight measurements were performed by filtration of 1 ml of culture through pre-weighed filters (0.45 µm) and washed three times with ultrapure sterilized water. Filters were dried at 60°C until constant weight and cell dry weight was determined.

Supernatant alcohol analysis

Supernatant alcohol composition was evaluated by headspace-solid-phase microextraction (HS-SPME) using a carbowax/divinylbenzene-coated fibre combined with gas chromatography-mass spectrometry (GC-MS) as described by Martins *et al.* [6]. In order to increase the sensitivity of the methodology, the samples were analysed in selective ion monitoring acquisition mode: farnesol (*m/z* 69, 107 and 161), nerolidol (*m/z* 69, 93 and 107), dodecanol (*m/z* 69, 83 and 111), phenylethanol (*m/z* 91 and 122), and isoamyl alcohol (*m/z* 55 and 77). These alcohols were identified by comparing GC retention times and mass spectra with those of pure standard compounds. A control analysis was performed with RPMI medium, and no interfering substances were found in the retention times of those compounds. The GC peak area data were used to: (i) compare alcohols' relative abundance in different *Candida* species (determining the peak areas in both species); (ii) estimate the compound level in the sample (comparing the GC peak area of each alcohol in the sample with that of one standard with a known concentration).

Biofilm formation on the surface of wells of microtiter plates

For the determination of the effect of alcohols on subsequent biofilm development, a rapid and robust method, the 96-well plate model [9] that allows testing of multiple parameters in the same experiment, was used. Due to the volatile nature of these alcohols and to avoid possible cross contaminations, each compound was assayed individually in a single microtitre plate.

Candida biofilms were formed on commercially available polystyrene, flat-bottomed, 96-well microtitre plates (Orange Scientific). Standardized cell suspensions (200 µl) were seeded into the wells, and the plates were incubated at 37°C and 130 rpm. After 3 h, the medium was aspirated, and the wells were washed twice with ultrapure sterile water to remove non-adherent cells.

For the study of alcohols' effect on subsequent biofilm formation of adhered cells, each alcohol diluted in RPMI medium (200 µl) was added to the adhered yeast cells. The plates were incubated at 37°C (130 rpm) for additional 24 h.

To evaluate alcohols' effect on further development of preformed biofilms, RPMI medium was added to adhered cells, and was renewed after 24 h of growth. At 48 h of incubation, the medium was removed, and the wells were washed as described above. Two hundred microlitres of fresh medium containing each alcohol at the desired concentrations were added to each well, and the plates were incubated for further 24 h.

Positive controls for biofilm formation consisted of unchallenged biofilms. Negative background controls for subsequent analysis consisted of non-seeded wells filled with RPMI medium. Solvent interferences were discarded including methanol controls. All wells contained at most 0.07% (v/v) of methanol. An agreement of more than 90% in biofilm parameters evaluated (see below) was observed for methanol treated and untreated sessile cells.

For each experiment, at the end of the incubation time, the wells were washed two times with ultrapure sterile water, and the biofilm development was estimated as described below.

Colony-forming units of cells in biofilms

In the assays performed, each alcohol was tested at the same concentrations in all *Candida* species, but the number of cells within biofilms may vary with *Candida* species. To estimate the number of colony forming units (CFU) of sessile cells before alcohol challenge, cells were scraped, after the washing procedure, and re-suspended in ultrapure sterile water in four steps: 3 × 100 µl plus 200 µl, in a total of 500 µl. Suspensions containing the biofilm cells were vortexed for 2 min. Viable counts were obtained by serial dilution in ultrapure sterile water, and plating on Sabouraud dextrose agar medium. After 24 h of incubation at 37°C, CFU were enumerated. The efficacy of biomass removal was assessed by crystal violet staining (see below) performed in each well.

Assessment of biofilm cells mitochondrial activity

Biofilm cells mitochondrial activity was determined through the quantification of the reduction of [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide] (XTT) [10]. Briefly, 200

µl of 100 µg/µl XTT (Sigma) and 10 µg/µl phenazine methosulphate (PMS; Sigma) were added to each well, containing washed biofilms. Then, the plates were incubated in the dark at 37°C and 130 rpm for 3 h. After that, cells were allowed to settle, and 150 µl of the supernatant was transferred to a new 96-well plate. Formazan salt formation was measured by absorbance reading at 490 nm (A_{490}) (Bio-Tek Synergy HT, Izasa). Controls were performed to check for interferences between XTT and the alcohols assayed, and no changes were detected in A_{490} readings. Samples exhibiting very intense color, yielding "offscale" absorbance values, were diluted as necessary before performing a second absorbance reading.

Assessment of biofilm total biomass (crystal violet assay)

Biofilm biomass was quantified using the crystal violet assay [11]. Briefly, after the washing procedure, air dried biofilms were fixed with 200 µl of 99% (v/v) methanol for 15 min. After methanol removal, the plates were left to air dry. Then, the biofilm within the wells was stained with 200 µl of crystal violet (VWR) diluted to 1% (v/v) during 5 min. Afterwards, each well was washed twice with ultrapure sterile water, air dried and destained with 200 µl of 33% (v/v) acetic acid (VWR). The resulting solution was transferred to a new microtiter plate, and the absorbance was measured at 570 nm (A_{570}). Samples exhibiting very intense color, yielding "offscale" absorbance values, were diluted as necessary before performing a second absorbance reading.

Reproducibility and statistical analyses

Biomass dry weight measurements were performed three times. Supernatant alcohol analysis was made with two replicates, with an agreement of results within ≤ 15%. CFUs were determined from four independent experiments with two technical replicates. Biofilm cells mitochondrial activity and biomass determinations were performed in sets of eight replicates, on at least three separate occasions.

Statistical analyses were performed using GraphPad Prism, version 5.00 software for Windows. Data normality was tested by the Kolmogorov-Smirnov method. The distribution of culture cell dry weight measurements was performed by Mann-Whitney test. The CFU of biofilms subjected to alcohols treatment were compared by Kruskal-Wallis, with a Dunn's post

test. Comparison between biofilm biomass and mitochondrial activity means of treated with untreated samples was performed by two-tailed unpaired t-test (confidence interval 95%). When the two sample standard deviations were not equal, Welch's correction to Student's t-test was used.

RESULTS

Qualitative evaluation of extracellular alcohols in *C. parapsilosis* and *C. tropicalis* supernatants

Candida species planktonic cultures biomass dry weight determined at the end of 24 h were not statistically different (median biomass dry weight of *C. parapsilosis* was 3.5 mg ml, and of *C. tropicalis* 3 mg/ml, P= 0.18). Cell free supernatants were analyzed by HS-SPME/GC-MS and the obtained alcohols profile is summarized in Table 3.3-1. Nerolidol was not detected in culture supernatants of both species. Farnesol and dodecanol were identified in both culture supernatants, and their concentration was below nanomolar (Table 3.3-1). The comparison of phenylethanol and isoamyl alcohol GC peak intensity in the two *Candida* species samples indicates that these compounds levels are approximately 20 × and 100 × higher in *C. tropicalis*, respectively, and for this *Candida* species their concentration is in the micromolar range (Table 3.3-1).

Table 3.3-1 Alcohols composition of *C. parapsilosis* and *C. tropicalis* supernatants- alcohols and the corresponding GC peak area ($\times 10^{-5}$) determined in samples and standards are shown-

Compound	Mean GC peak area ($\times 10^{-5}$)[a]		
	Standard (concentration)	*C. parapsilosis*	*C. tropicalis*
Farnesol	99 (12 nM)	2.8	1.5
Nerolidol	6 (4 nM)	n.d.	n.d.
Dodecanol	5.4 (0.06 nM)	3.9	10.9
Phenylethanol	710 (21 μM)	25.9	558.2
Isoamyl alcohol	390 (182 μM)	5.1	523.7

n.d., not detected; [a],with an agreement of results within ≤ 15%

Effect of the addition of synthetic alcohols in *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. tropicalis* biofilm development

The effect of farnesol, nerolidol, dodecanol, phenylethanol, and isoamyl alcohol on *Candida* species was individually evaluated on biofilm development of adhered cells (Fig.3.3-1) and mature biofilms (Fig.3.3-2).

The analysis of the number of culturable sessile cells at 3 h and 48 h revealed that: (i) *C. tropicalis* adhered cell population was higher than that of *C. albicans* (median log CFU of 5.2 vs 3.4, respectively, $P< 0.05$), and (ii) *C. dubliniensis* mature biofilms contained more culturable cells than those of *C. parapsilosis* (mean log CFU of 6.7 vs 5.8, respectively, $P< 0.05$).

Farnesol

Farnesol was used as a control for *C. albicans*, *C. dubliniensis* and *C. parapsilosis*. Results show that 150 μM farnesol only reduced *C. albicans* biofilm biomass by 50% (Fig.3.3-1 *A-II*), and *C. dubliniensis* mitochondrial activity by 38% (Fig.3.3-1 *A-I*) plus biofilm biomass by 64% (Fig.3.3-1 *A-II*) when added to the adhered cell population. This alcohol did not affect *C. parapsilosis* biofilm cells (Fig.3.3-1 *A* and Fig.3.3-2 *A*). These data is in accordance to previous reports [5, 7, 8]. Additionally, farnesol was tested on *C. tropicalis* sessile cells and no changes were detected on adhered cells (Fig.3.3-1 *A*) and mature biofilms (Fig.3.3-2 *A*) development.

Nerolidol

Nerolidol was assayed at three concentrations (1.5×10^{-3}, 1.5, and 150 μM) (Fig.3.3-1 *B* and Fig.3.3-2 *B*) and showed anti-biofilm activity against *C. parapsilosis* and *C. tropicalis* sessile cells.

C. parapsilosis adhered cells treatment with 1.5×10^{-3} and 1.5 μM nerolidol resulted in a reduction of mitochondrial activity ≥ 25% ($P< 0.05$) (Fig.3.3-1 *B-I*), whereas mitochondrial activity of mature biofilm cells was significantly decreased independently of the concentration tested (Fig. 3.3-2 *B-I*).

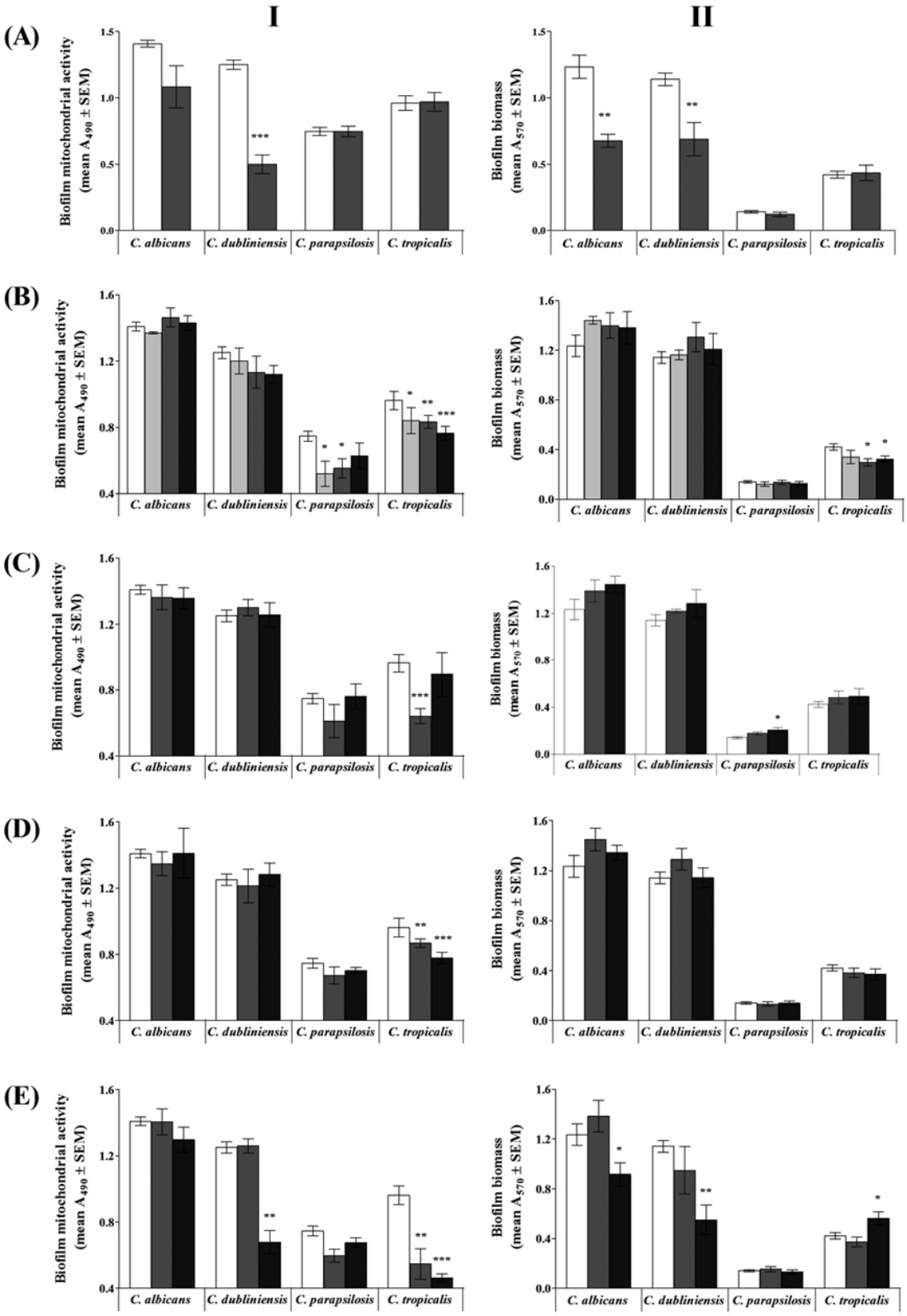

Fig.3.3-1. Effect of alcohols on *Candida* species adhered cells subsequent biofilm development. Biofilm mitochondrial activity, assayed by XTT reduction, (A_{490}) (***I***) and biofilm biomass, assayed by crystal violet staining (A_{570}) (***II***) were estimated 24 h after exposure to: farnesol (***A***) [0 (□) and 150 μM (■)], nerolidol (***B***) [0 (□), 1.5 × 10^{-3} (■), 1.5 (■), and 150 μM (■)], dodecanol (***C***) [0 (□), 2 × 10^{-3} (■), and 2 μM (■)], phenylethanol (***D***) [0 (□), 5 (■), and 500 μM(■)], and isoamyl alcohol (***E***) [0 (□), 46 × 10^{-3} (■), and 23 mM (■)]. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ for treated biofilms compared with untreated controls (unpaired t-test).

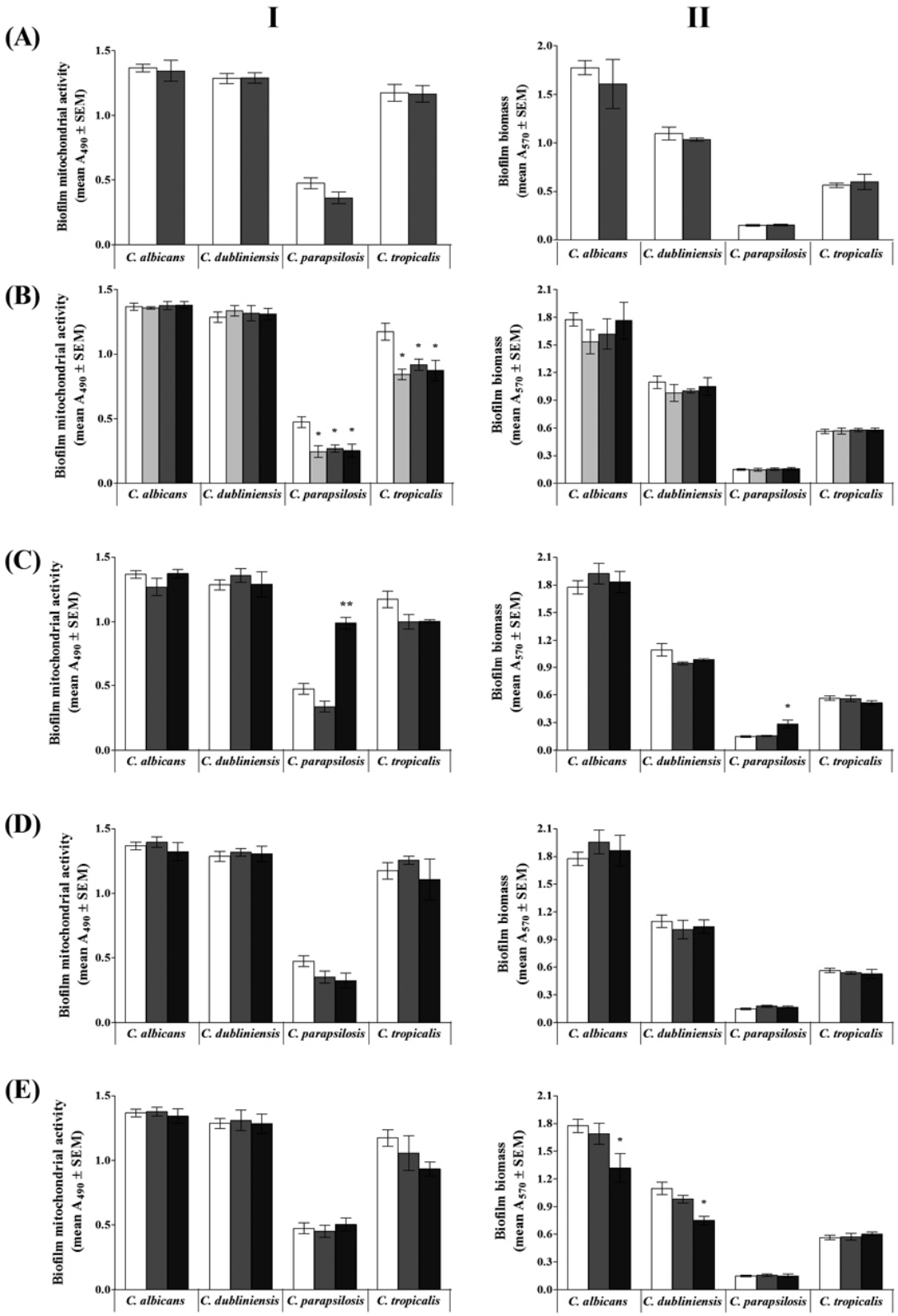

Fig.3.3-2. Effect of alcohols on *Candida* species mature biofilms subsequent biofilm development. Biofilm mitochondrial activity, assayed by XTT reduction, (A_{490}) (*I*) and biofilm biomass, assayed by crystal violet staining (A_{570}) (*II*) were estimated 24 h after exposure to: farnesol (*A*) [0 (□) and 150 μM (■)], nerolidol (*B*) [0 (□), 1.5 × 10^{-3} (■), 1.5 (■), and 150 μM (■)], dodecanol (*C*) [0 (□), 2 × 10^{-3} (■), and 2 μM (■)], phenylethanol (*D*) [0 (□), 5 (■), and 500 μM(■)], and isoamyl alcohol (*E*) [0 (□), 46 × 10^{-3} (■), and 23 mM (■)].*, $P < 0.05$, **, $P < 0.01$ for treated biofilms compared with untreated controls (unpaired t-test).

Biofilm formation by adhered *C. tropicalis* cells population was hampered by nerolidol. In fact, there was a statistically significant decrease in biofilm cells mitochondrial activity (Fig.3.3-1 *B-I*) and biofilm biomass (Fig.3.3-1 *B-II*) induced by nerolidol. In contrast, the addition of nerolidol (1.5×10^{-3} to 150 μM) to mature biofilms induced a reduction in mitochondrial activity during further development ($P < 0.05$) (Fig.3.3-2 *B-I*) but did not affect biofilm biomass (Fig.3.3-2 *B-II*).

Dodecanol

The effect of dodecanol was tested at concentrations of 2×10^{-3} and 2 μM (Fig.3.3-1 *C* and Fig.3.3-2 *C*). This compound elicited a pro-biofilm activity in *C. parapsilosis* but an anti-biofilm activity in *C. tropicalis* sessile cells.

Biofilm biomass of adhered *C. parapsilosis* cells was increased by 46% after the 24-h treatment with the highest alcohol concentration ($P = 0.04$) (Fig.3.3-1 *C-II*). Fig.3.3-2*C* shows that dodecanol (2 μM) concomitantly increased *C. parapsilosis* mature biofilm cells mitochondrial activity ($P = 0.004$) (Fig.3.3-2 *C-I*), and biofilm biomass ($P = 0.02$) (Fig.3.3-2 *C-II*) by more than 90% during the subsequent development.

C. tropicalis adherent cells 24-h treatment with 2×10^{-3} μM dodecanol resulted in a significant decrease in mitochondrial activity (33%) (Fig.3.3-1 *C-I*), without changes in biofilm biomass (Fig.3.3-1 *C-II*).

Phenylethanol

Phenylethanol at concentrations of 5 and 500 μM (Fig.3.3-1 *D* and Fig.3.3-2 *D*) showed anti-biofilm activity against *C. tropicalis* sessile cells. Specifically, the mitochondrial activity of *C. tropicalis* adhered cells (Fig.3.3-1 *D-I*) (but not mature biofilms, Fig.3.3-2 *D-I*) was significantly reduced in a dose dependent trend upon 24-h exposure to phenylethanol, with a 20% decrease for the highest concentration tested.

Isoamyl alcohol

Isoamyl alcohol concentrations of 46 × 10^{-3} and 23 mM used herein (Fig.3.3-1 *E* and Fig.3.3-2 *E*) elicited anti-biofilm activity against *C. albicans* and *C. dubliniensis*, and a heterogenous activity against *C. tropicalis* at early stages of development.

C. albicans adhered cells treatment with isoamyl alcohol 23 mM resulted in a 26% reduction of biofilm biomass (P= 0.02) during the subsequent development (Fig.3.3-1 *E-II*).

Biofilm formation by the adhered *C. dubliniensis* cells population was significantly reduced by 23 mM isoamyl alcohol in terms of mitochondrial activity (Fig.3.3-1 *E-I*) and biofilm biomass (Fig.3.3-1 *E-II*).

The 24-h challenge of *C. tropicalis* adhered cells with isoamyl alcohol (46 × 10^{-3} and 23 mM) induced a significant decrease in cellular mitochondrial activity (Fig.3.3-1 *E-I*). Unexpectedly, upon challenge with the highest isoamyl alcohol concentration assayed, biofilm biomass increased 30% (P= 0.04) (Fig.3.3-1 *E-II*).

DISCUSSION

Microbial supernatant media contain a variety of products that comprehend final fermentation products, temporarily released metabolites, secondary metabolites, and proteins [12]. In *Candida* field, a cohort of secreted alcohol molecules has been sparsely identified in culture supernatants, namely, farnesol [13], tyrosol [14], nerolidol, dodecanol, phenylethanol, and isoamyl alcohol [6]. In this study it was shown that *C. parapsilosis* and *C. tropicalis* secrete farnesol, dodecanol, phenylethanol, and isoamyl alcohol but not nerolidol, into the extracellular medium (Table 3.3-1). This suggests that, in general, the release of alcohols into the extracellular medium is a common trait of *Candida* species. However, farnesol and dodecanol concentration range in *C. parapsilosis* and *C. tropicalis* supernatant (below nanomolar) (Table 3.3-1) was lower than that previously described for *C. albicans* and *C. dubliniensis* (nanomolar) [6]. In contrast, the phenylethanol and isoamyl alcohol concentration range detected in *C. tropicalis* supernatant (within micromolar) (Table 3.3-1) was similar to that earlier described for *C. albicans* and *C. dubliniensis* [6]. Thus, although it cannot be excluded that the supernatant alcohol profiling may be dependent on experimental conditions [15], and strains used, data on

Table 3.3-1 suggests a heterogeneity in terms of the amounts of alcohols production among *Candida* species.

It has been suggested that the majority of the molecules secreted by microorganisms modulate physiological functions, although the observed effects are not always derived from a quorum sensing circuit but many times resultant from processes of compound metabolisation or detoxification [16]. This work examined the effect of the addition of commercial formulations of extracellular alcohols in *Candida* species biofilm formation (Fig.3.3-1 and Fig.3.3-2), as indicators of a role for secreted alcohols in biofilm development. In an attempt to ascertain the physiological relevance of the alcohols, this study was generally focused on the evaluation of the effect of physiological and slightly higher concentrations of secreted alcohols [6].

The alcohols treatment induced different types of effects, in terms of mitochondrial activity and biofilm biomass changes, for the specific strains evaluated. First, a 'dose-response' effect, which assumes that, as the dose of the effector increases, also does the biological response caused [17] as suggested by dodecanol (Fig.3.3-1 *C-II*, *C. parapsilosis*; Fig.3.3-2 *C-I* and *C-II*, *C. parapsilosis*), phenylethanol (Fig.3.3-1 *D-I*, *C. tropicalis*), and isoamyl alcohol (Fig.3.3-1 *E-I*, *C. dubliniensis*; Fig.3.3-1 *E-II*, *C. albicans*, *C. dubliniensis*, and *C. tropicalis*). Second, alcohols that induced the highest effect at the lower concentrations tested, which resembles the paradoxical effect [18]. An example shown herein include the biofilm development changes induced by dodecanol (Fig.3.3-1 *C-I*, *C. tropicalis*). Third, alcohols that triggered a similar quantitative effect despite the concentration used, as observed for nerolidol (Fig.3.3-1 *B-II*, *C. tropicalis*; Fig.3.3-1 *B-I* and Fig.3.3-2 *B-I*, *C. parapsilosis* and *C. tropicalis*), and isoamyl alcohol (Fig.3.3-1 *E-I*, *C. tropicalis*). Fourth, alcohols that induced a simultaneous change in mitochondrial cells activity and in biofilm biomass. Although only observed at alcohol supraphysiological concentrations, examples include: nerolidol (Fig.3.3-1 *B*, *C. tropicalis*), dodecanol (Fig.3.3-2 *C*, *C. parapsilosis*), isoamyl alcohol (Fig.3.3-1 *E*, *C. dubliniensis*), and farnesol (Fig.3.3-1 *A*, *C. dubliniensis*).

Overall, the alcohols assayed showed a moderate anti-biofilm activity, except dodecanol in *C. parapsilosis* (Fig.3.3-1 *C-II* and Fig.3.3-2 *C*), and isoamyl alcohol in *C. tropicalis* (Fig.3.3-1 *E-II*), that induced a pro-biofilm activity. However, it cannot be ruled out that these molecules elicit other effects in biofilms such as changes in morphology [19] or biofilm matrix [20]. The effects elicited by these alcohols did not appear to be compound specific, but rather dependent

on the biofilm development stage (Fig.3.3-1 and Fig.3.3-2). Specifically, although all the alcohols induced changes in further biofilm formation when added to adhered cells population (Fig.3.3-1), only nerolidol (Fig.3.3-2 *B-I*, *C. parapsilosis* and *C. tropicalis*) and dodecanol (Fig.3.3-2 *C-I* and *C-II*, *C. parapsilosis*) had influence on mature biofilms development.

During the last years the recognition of polymicrobial cultures has been increasing [21]. However, *Candida* species interactions within mixed species biofilms has been scarcely studied [22-24]. The production of diffusible alcohol molecules probably offers a strategy for communication between *Candida* species. As noted, nerolidol was not detected in *C. tropicalis* and *C. parapsilosis* supernatants (Table 3.3-1). Interestingly, this compound, even at *C. albicans* and *C. dubliniensis* physiologically relevant concentrations (nanomolar levels) elicited an anti-biofilm activity against these specific strains of *C. parapsilosis* and *C. tropicalis* at early (Fig.3.3-1 *B*) and late (Fig.3.3-2 *B*) stages of development. This suggests a competitive advantage for *Candida* species that are nerolidol producers, such as *C. albicans* and *C. dubliniensis* [6]. Additionally, it was also noticed that the levels of isoamyl alcohol and phenylethanol in *C. tropicalis* supernatants are higher than the observed for *C. parapsilosis* (Table 3.3-1). The exposure to these compounds did not affect *C. parapsilosis* biofilm but induced a decrease in *C. tropicalis* adhered cells mitochondrial activity at physiologically relevant levels (5 μM for phenylethanol and 43×10^{-3} mM for isoamyl alcohol). This suggests that these compounds have an antagonist effect in *C. tropicalis* biofilm development. However, further insights can be achieved by a time course characterization of alcohols production, to evaluate possible growth stage specificity, and by determining the profile of alcohols secretion by biofilm cells. Overall, additional studies to clarify the role of these molecules might include a much higher number of strains of each species, the recognition of compound specific receptors, and evaluation of transcriptional induced changes [12], the use of mutants that do not respond to the molecules and that respond to the molecules but do not increase the molecules production in response to the extracellular stimulus.

In conclusion, this study proved the extracellular secretion of farnesol, dodecanol, phenylethanol, and isoamyl alcohol by *C. parapsilosis* and *C. tropicalis* (as determined by HS-SPME GC-MS), and contributed to shed more light into cell-cell signaling molecules in *Candida*

species. Moreover, it was shown that physiological levels of these secreted alcohols play an effect in *C. parapsilosis* and *C. tropicalis* biofilm development, evidencing the complexity of biofilm formation regulation.

REFERENCES

1. Pfaller, M. A. & Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**, 133-163.
2. Coleman, D. C., Sullivan, D. J., Bennett, D. E., Moran, G. P., Barry, H. J., & Shanley, D. B. (1997) Candidiasis: the emergence of a novel species, *Candida dubliniensis*. *Aids* **11**, 557-567.
3. Ramage, G., Martinez, J. P., & Lopez-Ribot, J. L. (2006) *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* **6**, 979-986.
4. Eggimann, P., Garbino, J., & Pittet, D. (2003) Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect Dis* **3**, 685-702.
5. Ramage, G., Saville, S. P., Wickes, B. L., & Lopez-Ribot, J. L. (2002) Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* **68**, 5459-5463.
6. Martins, M., Henriques, M., Azeredo, J., Rocha, S. M., Coimbra, M. A., & Oliveira, R. (2007) Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot Cell* **6**, 2429-2436.
7. Laffey, S. F. & Butler, G. (2005) Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151**, 1073-1081.
8. Jabra-Rizk, M. A., Shirtliff, M., James, C., & Meiller, T. (2006) Effect of farnesol on *Candida dubliniensis* biofilm formation and fluconazole resistance. *FEMS Yeast Res* **6**, 1063-1073.
9. Pierce, C. G., Uppuluri, P., Tristan, A. R. *et al.* (2008) A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc* **3**, 1494-1500.
10. Henriques, M., Azeredo, J., & Oliveira, R. (2006) *Candida albicans* and *Candida dubliniensis*: comparison of biofilm formation in terms of biomass and activity. *Br J Biomed Sci* **63**, 5-11.
11. Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., & Svabic-Vlahovic, M. (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* **40**, 175-179.
12. Winzer, K., Hardie, K. R., & Williams, P. (2002) Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **5**, 216-222.
13. Weber, K., Sohr, R., Schulz, B., Fleischhacker, M., & Ruhnke, M. (2008) Secretion of *E,E*-farnesol and biofilm formation in eight different *Candida* species. *Antimicrob Agents Chemother* **52**, 1859-1861.
14. Cremer, J., Vatou, V., & Braveny, I. (1999) 2,4-(hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing in vitro. *FEMS Microbiol Lett* **170**, 319-325.

15. Ghosh, S., Kebaara, B. W., Atkin, A. L., & Nickerson, K. W. (2008) Regulation of aromatic alcohol production in *Candida albicans*. *Appl Environ Microbiol* **74**, 7211-7218.
16. Williams, P., Winzer, K., Chan, W. C., & Camara, M. (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* **362**, 1119-1134.
17. Hadley, C. (2003) What doesn't kill you makes you stronger. A new model for risk assessment may not only revolutionize the field of toxicology, but also have vast implications for risk assessment. *EMBO Rep* **4**, 924-926.
18. Wiederhold, N. P. (2007) Attenuation of echinocandin activity at elevated concentrations: a review of the paradoxical effect. *Curr Opin Infect Dis* **20**, 574-578.
19. Alem, M. A., Oteef, M. D., Flowers, T. H., & Douglas, L. J. (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell* **5**, 1770-1779.
20. Nobile, C. J., Nett, J. E., Hernday, A. D. *et al.* (2009) Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* **7**, e1000133.
21. Thein, Z. M., Seneviratne, C. J., Samaranayake, Y. H., & Samaranayake, L. P. (2009) Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* **52**, 467-475.
22. Kirkpatrick, W. R., Lopez-Ribot, J. L., Mcatee, R. K., & Patterson, T. F. (2000) Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *J Clin Microbiol* **38**, 902-904.
23. Thein, Z. M., Samaranayake, Y. H., & Samaranayake, L. P. (2007) Characteristics of dual species *Candida* biofilms on denture acrylic surfaces. *Arch Oral Biol* **52**, 1200-1208.
24. El-Azizi, M. A., Starks, S. E., & Khardori, N. (2004) Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *J Appl Microbiol* **96**, 1067-1073.

Chapter 3.4 Effect of exogenous administration of *Candida albicans* autoregulatory alcohols in a murine model of hematogenously disseminated candidiasis

Abstract

Candida albicans supernatants contain a mixture of autoregulatory alcohols. In vitro, when added individually or in combination, these alcohols inhibit the yeast to filamentous form conversion. Here we evaluate the in vivo effect of the exogenous administration of a Cocktail solution simulating the composition of alcohols present in a *C. albicans* culture supernatant (1 ml; 94 µM isoamyl alcohol, 70 µM 2-phenylethanol, 3.2 nM *E*-nerolidol and 18 nM *E,E*-farnesol) using the well established murine model of hematogenously disseminated candidiasis. Mice injected intraperitoneally with the Cocktail solution demonstrated increased survival and decreased organ fungal burden compared to control mice (injected with Vehicle only). Histological observations suggest that the Cocktail, to some extent, has an inhibitory effect on cell proliferation and filamentation within the tissues. These findings suggest that the exogenous administration of *C. albicans* autoregulatory alcohols displays a protective effect during disseminated candidiasis. This information could be harnessed to develop new strategies to combat deep-seated candidiasis.

The results presented over this chapter were:

(i) submitted as,

Martins, M., Henriques, M. Lopez-Ribot, J.L.,Oliveira, R. Effect of exogenous administration of *Candida albicans* autoregulatory alcohols in a murine model of hematogenously disseminated candidiasis.

(ii) presented as poster communication,

Martins, M., Lazzell, A.L., Henriques, M., Azeredo, J., Lopez-Ribot, J.L., Oliveira, R. 2009. Exogenous administration of a cocktail mimicking *Candida albicans* autoregulatory alcohols affects the progression of hematogenously disseminated candidiasis in mice. **4th Trends in Medical Mycology**, Athens, October 18th – 21st.

INTRODUCTION

Candida albicans is a normal commensal microorganism able to colonize both biotic and abiotic surfaces inside the host. However, it can take advantage of a human host's weakened defenses and cause life threatening infections [1]. This suggests that *C. albicans* has a high level of plasticity to sense and adapt to host's environmental conditions and associated microbiota [2]. Among the fungal factors, that might influence the pathogenesis of infection are chemical substances secreted biologically. Indirect evidence indicated that the treatment of rats oral epithelium with *C. albicans* 5 h supernatants increased cells mitotic indices [3]. In the last years a special attention has been given to *E*,*E*-farnesol (farnesol), a *C. albicans* autoregulatory alcohol. This alcohol is secreted and detected by *C. albicans* as a sensor of population density [4]. In vitro assays, carried out in culture flasks [4] and on several cell lines [5, 6], showed that farnesol regulates *C. albicans* filamentation. In vivo, the effect of the exogenous administration of farnesol was suggested to be dependent on the disease model. Specifically, this alcohol increases *C. albicans* virulence in the hematogenously disseminated candidiasis model, although it does not seem to regulate *C. albicans* morphology within the organs [7]. In contrast,farnesol has a local protective effect against oral candidiasis, which is associated with regulation of filamentation [8].

In addition, our group recently demonstrated that culture supernatants of *C. albicans* contain a mixture of autoregulatory alcohols that, besides farnesol, include isoamyl alcohol, 2-phenylethanol (phenylethanol), 1-dodecanol, and *E*-nerolidol (nerolidol) [9]. Moreover, in vitro, these molecules were capable of inhibiting *C. albicans* filamentation when used alone or in combination (simulating a 96-h culture supernatant) [9]. Thus, the aim of this study was to investigate the in vivo effect of the identified autoregulatory alcohols, through the exogenous administration of a solution simulating a *C. albicans* 96-h culture supernatant, in a murine model of hematogenously disseminated candidiasis.

MATERIAL AND METHODS

Chemicals

Isoamyl alcohol (Sigma), phenylethanol (Fluka), nerolidol (Fluka), and farnesol (Sigma) standards purity ranged from 85 to 99%. Pure compounds were stored according to suppliers' instructions. Stock solutions were prepared freshly in 5% (v/v) of ethanol in sterile pyrogen-free saline (Vehicle) before each experiment. In order to ensure compounds stability through the course of the experiments, appropriate in vitro *C. albicans* filamentation assays were performed concomitantly, following the methodology previously reported by our group [9]. The solution simulating *C. albicans* 96-h planktonic culture supernatant was composed of 94 µM isoamyl alcohol, 70 µM phenylethanol, 3.2 nM nerolidol, and 18 nM farnesol [9]. This solution will be referred to as "Cocktail".

Strain and culture conditions

C. albicans CAF2-1 strain [10] was used for these studies. Cultures were grown overnight in yeast extract-peptone-dextrose medium (US Biological) at 30°C. Cells were harvested by centrifugation and washed three times in sterile saline. After determination of cell number by haemocytometer counts, appropriate dilutions were made to prepare a suspension containing 1.5×10^6 cells/ml. Confirmation of the number and viability of cells was performed by plating serial dilutions on Sabouraud dextrose agar medium supplemented with 100 mg/l ampicillin.

Animal experiments

All animal experimentation was conducted following the National Institutes of Health guidelines for housing and care of laboratory animals and performed in accordance with Institutional regulations after pertinent review and approval by the Institutional Animal Care and Use Committee at The University of Texas at San Antonio. Disseminated candidiasis was induced in imunocompetent BALB/c female mice (6 to 8 week old) by lateral vein injections of 0.2 ml suspensions containing 3×10^5 colony forming units (CFU) of *C. albicans* strain CAF2-1. Immediately after infection mice were administered intraperitoneally (i.p.) with one time injection of 1 ml of the Vehicle (control group), or the Cocktail solution (experimental group). Total amounts of each autoregulatory alcohol per mouse were of 8 µg of isoamyl alcohol and

phenylethanol, 0.7 ng of nerolidol, and 4 ng of farnesol. In one set of experiments mice (eight per group) were monitored for survival, and in a second set of experiments animals (twelve per group) were sacrificed at pre-set times, 1-, 2-, and 3-days post-infection (four animals per group at each time point). At the time of death or sacrifice organs were removed for quantitative determination of fungal burdens. Briefly, the organs were recovered, weighted and homogenized, and appropriated dilutions were performed and plated onto Sabouraud agar medium for the determination of CFU counts. Under the conditions used in this study, the detection limits were of: 193 CFU/g heart, 103 CFU/g lung, 164 CFU/g spleen, 61 CFU/ gliver, 53 CFU/ g brain, and 146 CFU/g kidney. For statistical analysis, negative cultures were ascribed one half $\log_{10}$ lower than these limits.

Since the kidney represents the major target of infection in this model, one kidney per animal was processed for histology. Briefly, samples were fixed in 10% buffered formalin and stored at 4°C, embedded in paraffin, sectioned, and stained with Grocott- Gomori methenamine-silver [11] prior to microscopy evaluation.

Statistical analyses

Survival data and differences between groups were determined by the Kaplan-Meier log rank test. Organs CFU counts were standardized per gram of tissue, and statistically significant differences of the logarithmic values between groups were determined by the Mann-Whitney test. The GraphPad Prism, version 5.00 software for Windows was used for statistical analyses.

Results and Discussion

C. albicans commensal versus pathogenic lifestyles are intrinsically dependent on a very delicate balance between the microbe and its host, whose understanding is important to identify potential targets for treatment. In this sense, to gain insights into pathogen-host interactions through autoregulatory alcohols, the effect of a solution simulating the autoregulatory alcohols content of a *C. albicans* 96-h culture supernatant [9] was evaluated in vivo. Although several non-mammalian models have been studied in the last years, the mouse model of hematogenously disseminated candidiasis is a valuable experimental tool for the study of *C.*

albicans pathogenesis, since it reproduces many of the clinical features of the human disease [12].

The level of each individual autoregulatory alcohol in the Cocktail solution was below the toxic levels for mice. Specifically in mice, the i.p. lethal value for isoamyl alcohol is 232 mg/kg [13], the median lethal dose (LD50) value for phenylethanol is 400 g/kg [14], and for farnesol is 327 mg/kg [15]. To authors' knowledge, the i.p. LD50 has not been tested for nerolidol. However, the oral LD50 reported for nerolidol -≈ 10 g/kg- [16] is similar to that for farnesol -8.7 g/kg- [15], suggesting that the dose used here is not toxic. A 5% ethanol saline solution was used as a Vehicle to administer the Cocktail solution, corresponding to a total amount of 39 mg of ethanol per mouse. This ethanol dose is well below the reported toxicity levels [17, 18].

Exogenous administration of *C. albicans* autoregulatory alcohols affects the outcome of the infection

The effect of the exogenous administration of the Cocktail was examined in terms of survival (Fig.3.4-1). The median survival time of mice within the control group was 6 days (Fig.3.4-1 -

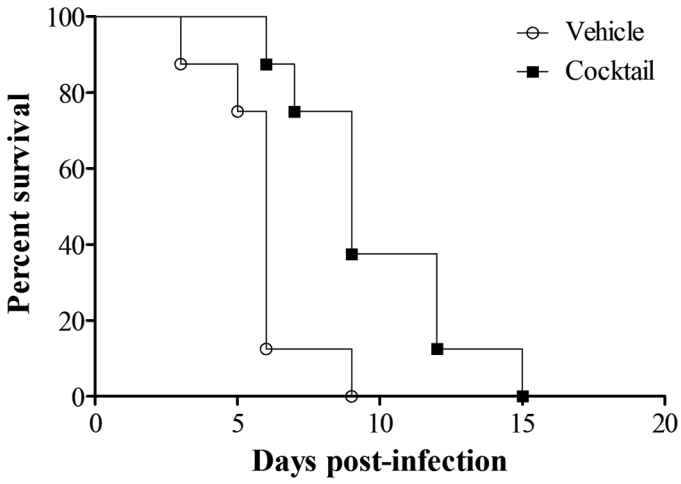

Fig.3.4-1. Effect of the administration of *Candida albicans* autoregulatory alcohols on mice survival. Groups of eight BALB/c mice were challenged intravenously with a suspension containing 3×10^5 CFU CAF2-1 strain/ mouse and 1 ml i.p. of Vehicle or Cocktail. Animals were monitored for their ability to survive infection. Survival data was analyzed by the Kaplan-Meier log rank test.

Vehicle). This is in general agreement with the expected survival for mice challenged with CAF2-1 strain only [19-21], evidencing the minor effect of Vehicle on mice survival. In contrast, death among mice challenged with *C. albicans* and 1 ml i.p. of the Cocktail solution occurred between days 6 and 15 post-infection (median survival time 9 days) (Fig.3.4-1, Cocktail). On day 6 post-infection the survival proportion of mice in the Cocktail group was 87.5% compared to only 12.5% in the Vehicle group. This indicates that the exogenous administration of the Cocktail significantly delays the time of death in comparison with untreated mice (P= 0.005).

Exogenous administration of *C. albicans* autoregulatory alcohols affects fungal load in deep organs

To evaluate whether the protective role observed for the Cocktail administration on survival correlated with the temporal progression of *C. albicans* infection, the fungal burden was determined for multiple organs at different times post-infection. On one hand, it is well established that in the hematogenously disseminated candidiasis model, *C. albicans* cells are cleared from the bloodstream within minutes post-infection [20, 22]. On the other hand, the autoregulatory alcohols pharmacokinetics is fast, with levels of compounds being undetectable in the bloodstream several hours after their administration [7, 13, 15]. Thus, the events mediated by the autoregulatory alcohols at early stages of the fungus–host interaction may determine the outcome of the disseminated candidiasis. In this sense, pre-set times sacrifices were performed 1-, 2-, and 3-days post-infection.

As seen in Table 3.4-1, on day 1 post-infection, *C. albicans* cells were recovered from all the organs analysed, and were similar between groups (P> 0.05). Generally, and consistent with prior reports in control animals treated with Vehicle only, fungal burdens decreased from day 1 to day 3 post-infection for all organs examined, except for brain and kidney, the two main targets organs in this model [20, 22]. Similar trends were observed for fungal burdens in heart, lung, spleen, and liver in the Cocktail-treated group. However, in marked contrast with the control group where fungal organ burdens were maintained at day 3 post-infection, brain fungal burden was decreased in Cocktail-treated animals, and at day 3 post-infection renal fungal burdens were significantly lower in the Cocktail-treated animals compared to control mice

Table 3.4-1. Effect of the administration of *C. albicans* autoregulatory alcohols on organs fungal burden. Groups of four BALB/c mice were challenged intravenously with a suspension containing 3×10^5 CFU CAF2-1 strain/ mouse and 1 ml i.p. of Vehicle and Cocktail. At day 1, 2, or 3 post-infection samples from the heart, lungs, spleen, liver, brain, and kidneys were recovered and each organ fungal burden monitored by determining the total CFU per gram of tissue. Data represents mean and standard deviation (SD), and the differences between groups were determined by the Mann-Whitney test.

Organ / Treatment	mean Log_{10} CFU/ g of tissue (SD)			P value		
	Pre-set times			Pre-set times		
	Day 1	Day 2	Day 3	Day 1 vs Day 2	Day 2 vs Day 3	Day 3 vs Day 1
Heart						
Vehicle	3.6 (0.1)	3.4 (0.005)	3.1 (0.3)	0.12	0.06	0.04
Cocktail	3.8 (0.1)	3.4 (0.2)	3.3 (0.2)	0.03	0.77	0.03
Lungs						
Vehicle	3.8 (0.2)	2.2 (0.3)	2 (0.8)	0.03	0.88	0.03
Cocktail	3.7 (0.1)	2.7 (0.2)	1.8 (0.6)	0.03	0.04	0.03
Spleen						
Vehicle	5.1 (0.2)	4.9 (0.2)	4.7 (0.2)	0.19	0.11	0.04
Cocktail	5.2 (0.2)	4.9 (0.1)	4.4 (0.1)	0.08	0.03	0.03
Liver						
Vehicle	4 (0.06)	3.3 (0.1)	3.2 (0.2)	0.03	0.6	0.03
Cocktail	3.8 (0.3)	3.2 (0.06)	2.8 (0.3)	0.03	0.03	0.03
Brain						
Vehicle	3.9 (0.2)	3.5 (0.4)	3.8 (0.4)	0.38	0.56	0.66
Cocktail	4.1 (0.2)	3.9 (0.2)	3 (0.4)	0.23	0.03	0.03
Kidney						
Vehicle	5.7 (0.2)	5.2 (0.3)	5.9 (0.4)	0.11	0.08	0.77
Cocktail	5.6 (0.4)	5.1 (0.8)	5.3 (0.2)	0.31	0.34	0.24

($P<0.05$) (Table 3.2-1). These decreased organ fungal burdens in brains and kidneys early after infection may explain the slower mortality observed for Cocktail-treated mice. However, we note that all animals treated with the mixture of autoregulatory alcohols eventually succumb to disseminated candidiasis, probably an indication of the short-time effects associated with a single administration of these molecules at the time of infection.

Exogenous administration of *C. albicans* autoregulatory alcohols affects fungal morphology and distribution in infected organs

We performed a histological analysis of kidneys recovered from both Cocktail- and Vehicle-treated (control) mice. As seen in Fig.3.4-2, this examination revealed the characteristic presence of filaments and large cellular aggregates in the kidneys of control mice three days post-infection (Fig. 3.4-2, *A* and *B*). However, examination of the kidneys recovered from mice treated with the mixture of autoregulatory alcohols at the same time point revealed the presence of mostly yeast cells with a more scattered distribution throughout the organs (Fig. 3.4-2, *C*). We note that cells displaying a filamentous morphology were detected in tissues from Cocktail-treated mice at time of death as infection progressed (Fig. 3.4-2, *D*).

In summary, our results indicate that the exogenous administration of a mixture containing *C. albicans* autoregulatory alcohols mimicking a 96h culture supernatant displays the capacity to modify the course and decrease the severity of hematogenously disseminated

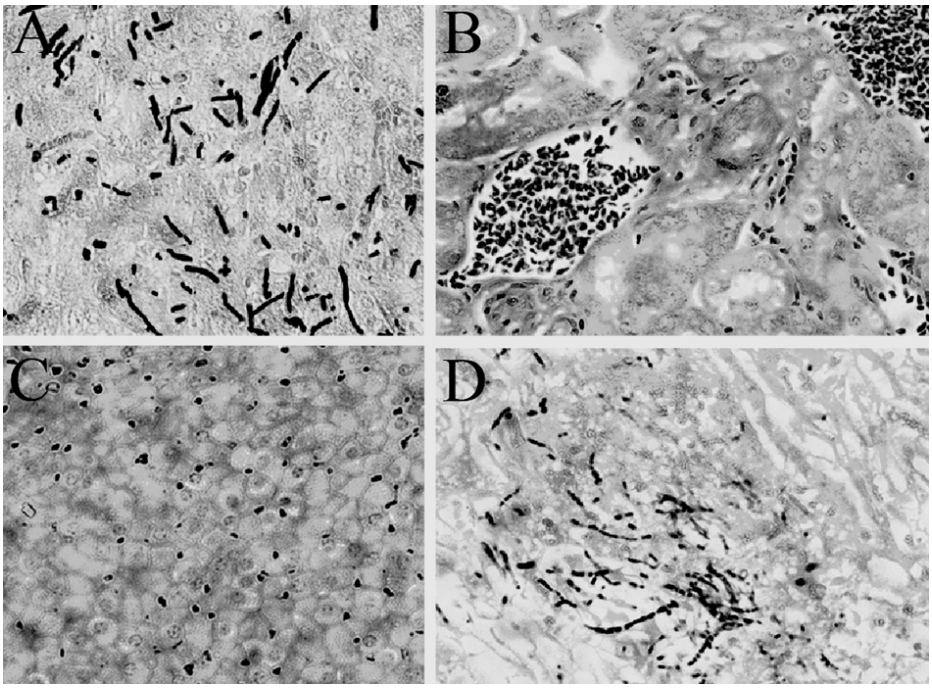

Fig.3.4-2. Histological analysis of kidneys retrieved from infected mice in the untreated (control) group (panels ***A*** and ***B***) and the Cocktail-treated group (panels ***C*** and ***D***). ***A***, filamentous forms are present in the kidney of an untreated mouse 3 days post-infection. ***B***, large aggregates of cells in the kidney of an untreated mouse 3 days post-infection. ***C***, scattered yeast cells in the kidney of a cocktail-treated mouse sacrificed 3 days after infection. ***D***, filamentous cells in the kidney of a Cocktail-treated mouse at time of death (7 days post-infection).

candidiasis in vivo: it increases survival, decreases organ fungal burden, and regulates morphogenetic conversions and global distribution patterns of fungal cells in infected tissues. These observations are consistent with similar results obtained in vitro (Martins *et al.*, 2007), but they seem somewhat contradictory to results previously reported for single farnesol administration in a similar in vivo model of candidiasis [7]. However, the differences in the mouse and *C. albicans* strains, and inoculum load [20, 23], or in the Vehicle used to deliver the alcohol in both studies may partially be responsible for this discrepancy.

Although many questions remain unresolved, particularly whether these autoregulatory alcohols are produced in vivo, and to which extent host factors or environments regulate their production during infection, overall the data presented here provides additional insight into the role of these autoregulatory alcohols in vivo and point towards the possibility of novel therapeutic strategies to combat disseminated candidiasis.

REFERENCES

1. d'Enfert, C. (2009) Hidden killers: persistence of opportunistic fungal pathogens in the human host. *Curr Opin Microbiol* **12**, 358-364.
2. Rozell, B., Ljungdahl, P. O., & Martinez, P. (2006) Host-pathogen interactions and the pathological consequences of acute systemic *Candida albicans* infections in mice. *Curr. Drug Targets.* **7**, 483-494.
3. Reed, M. F., Scragg, M. A., Williams, D. M., & Soames, J. V. (1990) In vivo effects of *Candida albicans* products on rat oral epithelium. *J. Oral Pathol. Med.* **19**, 326-329.
4. Hornby, J. M., Jensen, E. C., Lisec, A. D. *et al.* (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982-2992.
5. Saidi, S., Luitaud, C., & Rouabhia, M. (2006) In vitro synergistic effect of farnesol and human gingival cells against *Candida albicans*. *Yeast* **23**, 673-687.
6. Abe, S., Tsunashima, R., Iijima, R. *et al.* (2009) Suppression of anti-*Candida* activity of macrophages by a quorum-sensing molecule, farnesol, through induction of oxidative stress. *Microbiol Immunol* **53**, 323-330.
7. Navarathna, D. H., Hornby, J. M., Krishnan, N., Parkhurst, A., Duhamel, G. E., & Nickerson, K. W. (2007) Effect of farnesol on a mouse model of systemic candidiasis: a DPP3 knock out mutant of *Candida albicans*. *Infect Immun* **75**, 1609-1618.
8. Hisajima, T., Maruyama, N., Tanabe, Y. *et al.* (2008) Protective effects of farnesol against oral candidiasis in mice. *Microbiol Immunol* **52**, 327-333.

9. Martins, M., Henriques, M., Azeredo, J., Rocha, S. M., Coimbra, M. A., & Oliveira, R. (2007) Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot Cell* **6**, 2429-2436.
10. Fonzi, W. A. & Irwin, M. Y. (1993) Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717-728.
11. Grocott, R. G. (1955) A stain for fungi in tissue sections and smears using Gomori's methenamine-silver nitrate technic. *Am J Clin Pathol* **25**, 975-979.
12. Capilla, J., Clemons, K. V., & Stevens, D. A. (2007) Animal models: an important tool in mycology. *Med Mycol* **45**, 657-684.
13. (1978) Isoamyl alcohol. *Food Cosmet Toxicol* **16**, 785-788.
14. (1975) Phenylethyl alcohol. *Food Cosmet Toxicol* **13**, 903-904.
15. Lapczynski, A., Bhatia, S. P., Letizia, C. S., & Api, A. M. (2008) Fragrance material review on farnesol. *Food Chem Toxicol* **46**, S149-S156.
16. Lapczynski, A., Bhatia, S. P., Letizia, C. S., & Api, A. M. (2008) Fragrance material review on nerolidol (isomer unspecified). *Food Chem Toxicol* **46**, S247-S250.
17. Schechter, M. D. & Meehan, S. M. (1995) The lethal effects of ethanol and cocaine and their combination in mice: implications for cocaethylene formation. *Pharmacol Biochem Behav* **52**, 245-248.
18. Camarini, R., Andreatini, R., & Monteiro, M. G. (1995) Prolonged treatment with carbamazepine increases the stimulatory effects of ethanol in mice. *Alcohol* **12**, 305-308.
19. Banerjee, M., Thompson, D. S., Lazzell, A. *et al.* (2008) UME6, a novel filament-specific regulator of *Candida albicans* hyphal extension and virulence. *Mol Biol Cell* **19**, 1354-1365.
20. MacCallum, D. M. & Odds, F. C. (2005) Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48**, 151-161.
21. Saville, S. P., Lazzell, A. L., Chaturvedi, A. K., Monteagudo, C., & Lopez-Ribot, J. L. (2009) Efficacy of a genetically engineered *Candida albicans* tet-NRG1 strain as an experimental live attenuated vaccine against hematogenously disseminated candidiasis. *Clin Vaccine Immunol* **16**, 430-432.
22. Saville, S. P., Lazzell, A. L., Monteagudo, C., & Lopez-Ribot, J. L. (2003) Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot Cell* **2**, 1053-1060.
23. Ashman, R. B. & Papadimitriou, J. M. (1987) Murine candidiasis. Pathogenesis and host responses in genetically distinct inbred mice. *Immunol Cell Biol* **65**, 163-171.

CHAPTER 4

Insights into *Candida* world: *Candida* species in a clinical context

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Oral *Candida* carriage of patients attending a dental clinic in Braga, Portugal

Abstract

The ability of the *Candida* species to colonize surfaces can be considered a risk factor for oral infection. The aim of this work was to establish oral *Candida* carriage in patients attending a dental clinic in Braga, Portugal. A total of 97 patients were analysed. Swab samples were collected, and directly cultured in CHROMagar Candida. Representative yeasts were identified by polymerase chain reaction. From the samples analysed 54.6% (*n*=53) were *Candida* positive, and *Candida albicans* was the most frequently isolated species, accounting for 79% of all the species identified. Non-*C. albicans Candida* (NCAC) species recovered included *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, and *Candida guilliermondii*. There was a lack of association between the presence of *C. albicans*, and NCAC species, and age, gender or prostheses wearing in this population. In 17% of the cases (*n*=9) polymicrobial cultures, with two different *Candida* species, were identified. This study shows a high *Candida* carriage rate among this population, thus pointing to the relevance of an accurate diagnostic approach in *Candida* species identification.

The results presented over this chapter were:

(i) published as,

Martins, M., Henriques, M., Ribeiro, A.P., Fernandes, R., Gonçalves, V., Seabra, A., Azeredo, J., Oliveira R., Oral *Candida* carriage of patients attending a dental clinic in Braga, Portugal. **Rev Iberoam Micol**. In press.

(ii) presented as oral communication,

Martins, M.I., Henriques, M., Ribeiro, A.P., Fernandes, R., Gonçalves, V., Seabra, A., Azeredo, J., Oliveira, R. 2008. A study of oral candidiasis in a Portuguese dental clinic during a one year period. **ASM Conferences 9th Candida and Candidiasis**, March 24th – 28th 2008, New Jersey, USA, S2:3. p.19

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INTRODUCTION

Colonization of the oral cavity by *Candida* species was defined as the acquisition, and maintenance of yeast cells without clinical signs. This process entails *Candida* species acquisition, growth, and removal [1].

Within the yeast oral community *Candida albicans* is the most frequently found (47–75% of the yeasts isolated) [2]. However, other yeast species have been increasingly identified, such as Non-*C. albicans Candida* (NCAC) species (*Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis*, *Candida dubliniensis*, and *Candida guilliermondii*) *Saccharomyces cerevisiae*, *Trichosporon* species, and *Yarrowia lipolytica* [3-6].

Although the presence of *Candida* species is not an indication of disease, the ability of the yeasts to overcome the host clearance mechanisms and to colonize surfaces can be considered a risk factor for oral infection. The balance between *Candida* colonization, and candidiasis rely on the balance between pathogen characteristics (e.g., production of adhesins, secreted aspartyl proteinases), and host factors [7]. Host local predisposing conditions comprise: (i) reduced saliva secretion, (ii) epithelial changes, and local mucosal diseases, (iii) changes in commensal flora, (iv) high carbohydrate diet, and (v) denture wearing. Additionally, host systemic factors have also been associated with *Candida* oral colonization, and include: (i) age, (ii) tobacco smoking (iii) endocrine disorders, including diabetes, hypothyroidism, hyperparathyroidism, (iv) rheumatic diseases, (v) nutritional deficiencies (iron or folate deficiencies), (vi) immunosuppressive conditions, such as chemotherapy, deficiencies of humoral or cell-mediated immunity, human immunodeficiency virus infection, and acquired immunodeficiency syndrome, and (vii) drugs: broad-spectrum antibiotics, and corticosteroids [2, 5, 8, 9].

To the authors' knowledge oral *Candida* carriage prevalence, and aetiology studies were not yet performed in the Portuguese population. Thus, the main objective of this study was to evaluate oral *Candida* carriage in patients attending a dental clinic in Braga, Portugal.

PATIENTS AND METHODS

Candida type strains

For quality control purposes, the following *Candida* type strains were used in the identification procedures of *Candida* isolates: *C. albicans* CECT 1472, *C. dubliniensis*, strain provided by Biognostica from United Kingdom National External Quality Assessment Service, *C. glabrata* ATCC 2001, *C. guilliermondii* ATCC 6260, *C. kefyr* ATCC 204093, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750. These strains were maintained on Sabouraud dextrose agar.

Patients

Samples were collected from a total of 97 asymptomatic individuals (77 females, and 20 males) attending a dental clinic in Braga, Portugal over a 12-month period (May 2005 to 2006). Each of the potential subjects was informed of the aims, and methods of the study, and anticipated benefits, and potential risks, according to the World Medical Association Declaration of Helsinki. Data on patient age, oral hygiene habits, health status, medications, and prosthesis wearing were collected.

Sample collection

Samples were collected by passing a sterile swab (UNI-TER, MEUS, Padua, Italy) across the oral mucosa: tongue, hard palate, and gums, and replaced in its sterile container tube. Samples were kept at 4°C, and analysed within 24 h.

Candida species identification

Medium for the primary isolation

CHROMagar™ Candida medium (CHROMagar, Paris, France) was prepared according to the manufacturer's instructions. The swab was inoculated into CHROMagar Candida medium rotating the swab head on the surface of the medium. The plates were incubated at 37°C for 48 h. Colony morphology, and colour description were assigned in a standard manner by a single investigator. Presumptive species identification was performed according to Odds and Bernaerts

[10]. At least one colony exhibiting each colour was streaked into a new CHROMagar Candida plate, and then cryopreserved.

Molecular identification

Yeast DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Lisbon, Portugal) according to the manufacturer's instructions. Genomic DNA content was determined by spectrophotometry readings at 260 nm. Aliquots of 10 µl were analysed by electrophoresis in a 0.8% agarose (Bio-Rad, Lisbon, Portugal) gel in 1 × Tris-borate-EDTA (TBE) buffer (Bio-Rad, Lisbon, Portugal), and visualized with a ultraviolet (UV) transilluminator, after ethidium bromide (Bio-Rad, Lisbon, Portugal) staining (0.5 mg/ml).

To assess the *Candida* speciation, a polymerase chain reaction method (PCR) previously described [11] was followed. This method uses primer mixtures of the *Candida* DNA topoisomerase II genes (Table 4-1). In a multiplex PCR strategy it allows the identification of *C. albicans*, *C. guilliermondii,* and *C. parapsilosis* using primer set A (Table 4-1) and *C. dubliniensis*, *C. krusei*, *C. kefyr,* and *C. glabrata* using primer set B (Table 4-1). *C. tropicalis* is identified in a third PCR reaction using a single pair of primers (Table 4-1). PCR amplification was performed in 25 µl volume consisting of: 1 × PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20, and 25 mM $MgCl_2$) (Bioron, Porto, Portugal); dNTP mixture (200 µM each) (Bioron, Porto, Portugal); primer mixture (300 nM each); 1.25 U Taq DNA polymerase (Bioron, Porto, Portugal); 10–100 ng genomic DNA template; the remaining volume consisted of sterilized ultrapure water. PCR was carried out in a MyCycler thermal cycler (Bio-Rad, Lisbon, Portugal) under the following cycling conditions: 35 cycles of 30 s at 94°C, 15 s at 57°C, and 45 s at 65°C, after a 10-min initial period of DNA denaturation, and enzyme activation at 94°C. One blank reaction was performed simultaneously per every 10 tests run by replacing the template DNA by sterilized ultrapure water. DNA from type strains was also included in each reaction as positive and negative controls. An aliquot of 15 µl of each PCR product was analysed by electrophoresis in a 1.2% agarose gel in 1 × TBE buffer. Fragments were visualized by ethidium bromide staining (0.5 mg/ml) with a UV transilluminator. The size of the amplified DNA fragments was determined by comparison with a 100-bp DNA marker (Bioron, Porto, Portugal). All the isolates whose presumptive identification in CHROMagar

Table 4-1. Primers sets and species specific primers used in this study (as described by Kanbe *et al.* [11])

Primers sets	Target species	Forward primer sequence (name)	Reverse primer sequence (name)	Expected PCR product size (bp)
A[a]	*C. albicans*	5'-TTGAACATCTCCAGTTTCAAAGGT-3' (CABF59)	5'-AGCTAAATTCATAGCAGAAAGC-3' (CADBR125)	665
	C. guilliermondii	5'-CCCAAAATCACAAAGCTCAAGT-3' (CGLF41)	5'-TACGACTTGAAGTTGCGAATTG-3' (CGLR61)	205
	C. parapsilosis	5'-GGACAACATGACAAAAGTCGGCA-3' (CPPIIF41)	5'-TTGTGGTGTAATTCTTGGGAG-3' (CPPIIR69)	310
B[b]	*C. dubliniensis*	5'-AAATGGGTTTGGTGCCAAATTA-3' (CDBF28)	5'-GTTGGCATTGGCAATAGCTCTA-3' (CDBR110)	816
	C. krusei	5'-GAGCCACGGTAAAGAATACACA-3' (CKSF35)	5'-TTTAAAGTGACCCGGATACC-3' (CKSR57)	227
	C. kefyr	5'-CTTCCAAAGGTCAGAAGTATGTCC-3' (CKFF35)	5'-CTTCAAACGGTCTGAAACCT-3' (CKFR85)	532
	C. glabrata	5'-CCCAAAAATGGCCGTAAGTATG-3' (CGBF35)	5'-ATAGTCGCTACTAATATCACACC-3' (CGBR103)	674
C[c]	*C. tropicalis*	5'-CTGGGAAATTATATAAGCAAGTT-3' (CTPIIF36)	5'-TCAATGTACAATTATGACCGAGTT-3' (CTPIIR121)	860

In the original report [11] primers sets had the designation of [a]PsI, [b]PsII, and [c]PsIII

Candida did not correspond to the molecular identification were re-tested by two independent researchers in a blind assay.

Statistical analysis

CHROMagar Candida medium sensitivity was calculated as: [# true positives × 100 / (# true positives + # false negatives)], and specificity as: [# true negatives × 100 / (# true negatives + # false positives)]. Statistical analysis was performed using GraphPad Prism, version 5.00 software for Windows. Data was analysed using two-tailed chi-square test or Fischer test to measure association between *Candida* species distribution within groups. A statistical confidence interval of 95% was established.

RESULTS

Candida species identification

Presumptive identification of yeasts clinical isolates was based on their colour on CHROMagar Candida medium. Samples were processed in parallel with *C. albicans*, *C. krusei*, and *C.*

tropicalis type strains that presented the expected colours on CHROMagar Candida medium: green, pink, and blue, respectively.

For each culture, representative isolates were identified by PCR using *Candida* specific primers pairs for the genomic sequences of DNA topoisomerase II gene [11]. Reference strains DNA was included in each assay as control. DNA of *C. albicans* (Fig.4-1 *A*, lane 1), *C. guilliermondii* (Fig.4-1 *A*, lane 3), and *C. parapsilosis* (Fig.4-1 *A*, lane 4) were amplified using primer set A (Table 4-1). Primer set B (Table 4-1) allowed the identification of *C. glabrata* (Fig.4-1 *1B*, lane 2), *C. dubliniensis*, *C. krusei*, and *C. kefyr* reference strains (data not shown). Finally, the set of primers C (Table 4-1) allowed the identification of *C. tropicalis* reference strain

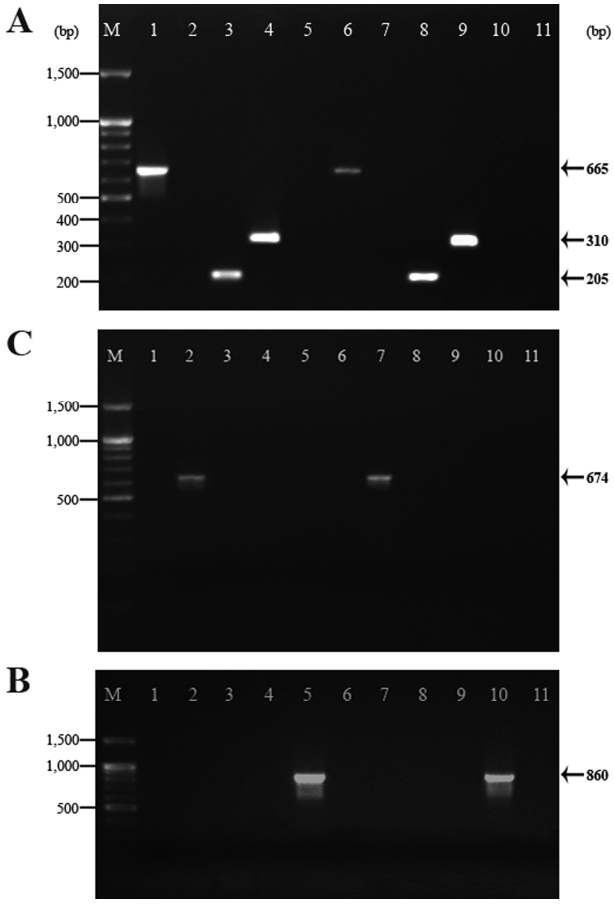

Fig.4-1. *Candida* species-specific amplification of DNA topoisomerase II fragments. Genomic DNA was amplified using different sets of species-specific primers (from ***A*** to ***C***), in accordance with Table 1. Lanes: ***M***,100-bp DNA marker and their molecular size in bp is indicated in the left margin; ***1***. *C. albicans* CECT 1472; ***2***. *C. glabrata* ATCC 2001; ***3***. *C. guilliermondii* ATCC 6260; ***4***. *C. parapsilosis* ATCC 22019; ***5***. *C. tropicalis* ATCC 750; ***6–10***. example of clinical isolates of each species; ***11***. blank. *C. dubliniensis*, *C. krusei* ATCC6258, and *C. kefyr* ATCC 204093 identification was omitted for simplicity. Arrows on the right indicate molecular weight of the amplified products.

(Fig.4-1 *1C,* lane 5). For all *Candida* species the amplicon size obtained (Fig.4-1) was as expected (Table 4-1). Clinical isolates identification was based on the comparison of the size of the amplified DNA products (assessed by the DNA ladder), with the respective type strain PCR product. Fig.4-1 shows an example of the identification of an isolate of *C. albicans* (lane 6), *C. glabrata* (lane 7), *C. guilliermondii* (lane 8), *C. parapsilosis* (lane 9) and *C. tropicalis* (lane 10).

CHROMagar Candida phenotypic characteristics of the *Candida* species identified by PCR are specified in Table 4-2. As expected, *C. glabrata*, and *C. parapsilosis* did not present a distinguishable colour in this medium. Using PCR as a standard method, and considering the CHROMagar Candida identifying colours claimed by the manufacturer, CHROMagar Candida sensitivity, and specificity for *C. albicans* were found to be 97.9%, and 83.3%, respectively. For *C. tropicalis* CHROMagar Candida sensitivity was 66.7%, and specificity 100%.

Candida species carriage

From the 97 patients evaluated, 53 were identified as oral *Candida* carriers: 81.1% were females (*n*=43), and 18.9% males (*n*=10) with ages ranging from 28 to 91 years old (mean=61, and median=62 years old). Prosthesis wearers accounted for 84.9% of the individuals (*n*=45).

C. albicans was identified in 79% of the samples being the predominant *Candida* species. Additionally, *C. parapsilosis* comprised 6.5% of the isolates, followed by *C. glabrata* (4.8%), *C. tropicalis* (3.2%), and *C. guilliermondii* (1.6%). Five percent of the CHROMagar Candida positive samples (Table 2) were not identified. The distribution of *Candida* isolates within gender, and age groups is presented in Table 4-3. There was no association between *C. albicans*, and NCAC

Table 4-2. Colour of colonies on CHROMagar Candida medium for species identified by PCR

Species	# Colony colour		
	Green	Blue	Pink
C. albicans	48	-	1
C. parapsilosis	-	-	4
C. glabrata	-	-	3
C. tropicalis	1	2	-
C. guilliermondii	-	-	1
Unidentified	1	-	2

Table 4-3. Frequency of distribution of *Candida* species isolated and patients' characteristics

Patients characteristics	Species[a] frequency, % (#)					
	C. albicans	NCAC species				
		C. parapsilosis	*C. glabrata*	*C. tropicalis*	*C. guilliermondii*	Unidentified
Gender						
Female	80 (40)	6 (3)	4 (2)	4 (2)	0 (0)	6 (3)
Male	75 (9)	8.3 (1)	8.3 (1)	0 (0)	8.3 (1)	0 (0)
Age (years)						
≤ 54	82.6 (19)	4.3 (1)	4.3 (1)	4.3 (1)	0 (0)	4.3 (1)
55-79	75.9 (22)	6.9 (2)	3.4 (1)	3.4 (1)	3.4 (1)	6.9 (2)
≥ 80	80 (8)	10 (1)	10 (1)	0 (0)	0 (0)	0 (0)

[a], PCR identification

species carriage within (i) gender (P=0.7), (ii) the age groups defined (P=0.83) or (iii) prosthesis wearing (P=1).

Seventeen percent of the individuals (*n*=9) presented more than one *Candida* species per sample (Table 4-4). These individuals (seven females, two males) had a mean age of 58 years old (range=38-88 years old). The percentage of patients wearing prostheses in this sub-population (88.9%) was similar to the observed in the population studied.

Table 4-4. Number of patients with more than one *Candida* species

Species[a]	#patients
C. albicans–C. glabrata	3
C. albicans–C. tropicalis	2
C. albicans–C. parapsilosis	1
C. albicans–unidentified	2
C. parapsilosis–C. guilliermondii	1

[a], PCR identification

Discussion

Motivation for microbiological tests in the field of oral medicine includes diagnosis, choice of therapy, treatment control, and risk evaluation [12]. This study focused on diagnostic, and risk evaluation.

The diagnosis approach used herein included a primary isolation on a chromogenic medium (CHROMagar Candida) followed by *Candida* species identification using a previously described PCR method [11]. The CHROMagar Candida phenotypic characteristics of the *Candida* species identified by PCR are specified in Table 4-2. In comparison with PCR, there was a lower sensitivity, and specificity of CHROMagar Candida in the identification of *C. tropicalis*, and of sensitivity regarding *C. albicans*, also described by other authors [13]. In fact, the low sensitivity shown by *C. tropicalis* to CHROMagar Candida (66.7%) is due to the fact that one isolate further identified as *C. tropicalis* presented green colour (Table 4-2), instead of the expected blue one. *C. tropicalis* isolates developing dark pink [14], lavender [13, 15], and white colour [13] on CHROMagar Candida have also been reported. Regarding *C. albicans*, two isolates that developed green colour on CHROMagar Candida were not identified as *C. albicans* (Table 4-2). In addition, one of the isolates, further identified as *C. albicans*, developed pink colour in CHROMagar Candida. In accordance, some literature reports show that *C. albicans* isolates can develop atypical colours in CHROMagar Candida which include pink [16], white [13], blue or lavender [15]. It should be noted that in the same plates containing these atypical strains, isolates of *C. albicans*, and *C. tropicalis* with the expected colour phenotype were identified.

Additionally, three yeast isolates were not identified by the PCR method using specific primers for the most common species (Table 4-2). A comparison between literature reporting yeast species frequently isolated from the oral cavity [5], and colours developed by *Candida* species in CHROMagar Candida [10], suggests that the unidentified isolate developing green colour might be a *Trichosporon* species, and the pink isolates might be: *Candida famata*, *Candida inconspicua*, *Candida lusitaniae*, *Candida norvegensis*, *Candida pelliculosa* or *S. cerevisiae*.

Results presented herein evidence that CHROMagar Candida medium failed to identify some yeast isolates, and that species identification should be supported by other methods, such as the molecular ones. Nevertheless, this medium facilitates the recognition of polymicrobial species in cultures, as exemplified in Table 4-4.

The analysis of the epidemiological literature on the recovery of *Candida* species from the oral cavity is not clear concerning factors determining colonization. The reasons of such variability may include different patient selection criteria, collection data period, geographic

region in which the patients live, sampling collection methods, and methodology used for sample analysis.

In the present study, the prevalence of yeasts isolated was 54.6%, and between 41% to 67% in previous studies [3, 6, 17-19]. However, due to the irregular distribution of *Candida* in the oral cavity [20], it cannot be discarded that swab samples can yield false-negative results, and thus a misclassification of true carriers as non–carriers. The *Candida* carriage frequency observed herein was: *C. albicans* > *C. parapsilosis* > *C. glabrata* > *C. tropicalis* > *C. guilliermondii* (Table 4-3), with NCAC species standing for 21% of the total *Candida* species. The increased prevalence of *C. parapsilosis* within NCAC species was also observed in Portuguese patients with fungaemia [21], suggesting that *C. parapsilosis* might be an important fungal pathogen in Portugal. Nevertheless prevalence of *C. tropicalis* [18], *C. parapsilosis* [22], *C. famata* [4] or *C. glabrata* [6, 19] over other NCAC species in the oral cavity has been shown, and such variation may be due to patient age or underlying disease.

The distribution of *Candida* isolates within gender, and age groups are presented in Table 3. In the current study there was no association between *C. albicans* vs NCAC species carriage within gender or age, likewise the observed by other authors [23]. Nevertheless, NCAC species recovered from samples of patients with more than 80 years old were exclusively *C. parapsilosis*, and *C. glabrata* (Table 4-3), suggesting an association between this age group, and these *Candida* species. Even so, as only two NCAC species have been isolated in this age group, an increase in sample size would be necessary to establish a conclusive association.

Earlier studies scarcely report the identification of mixed *Candida* cultures. However, in recent years, researchers became aware of it, and the refinement of identification procedures allowed the discrimination of multi-*Candida* species in culture. As observed in other studies [3, 6, 19, 24-26], the most common association found herein was *C. albicans* plus *C. glabrata* (Table 4-4). Nevertheless, the epidemiological data available report the association between other *Candida* species [3, 4, 6, 19, 24-26]. It is interesting to note that in the current study *C. parapsilosis* was the only NCAC species identified that was not exclusively co–isolated with other *Candida* species (Tables 4-3, and 4-4). In fact, other authors have reported that the colonization with NCAC species, as the sole species, is lower when compared with its co–colonization with other species [19]. This suggests that multi–species colonization may support the maintenance of the oral NCAC population contributing to increased interactions with

molecules, and surfaces in the oral cavity. In fact, it was shown that the intensity of colonization by more than one *Candida* species was higher than the observed with single species [19]. However, when host natural defences decay, the benign colonization can develop into oral candidiasis, and antifungal therapy may support NCAC species emergence as the sole detectable species from oral lesions. In line with this, results from the ARTEMIS DISK Global Antifungal Surveillance Program [27-29] show that *Candida* species resistance to fluconazole can be ranked as follow: *C. glabrata*, 16%, *C. guilliermondii*, 13%, *C. tropicalis*, 2.6%, *C. parapsilosis*, 2.4%, and *C. albicans*, 1.2%. These findings suggest that multi–species carriers might be at higher risk, than the mono–species carriers, of developing oral candidiasis, and of being resistant to antifungal therapy.

According to the World Medical Association Declaration of Helsinki, purposes of research involving human individuals might be the advance in prophylactic, diagnostic, and therapeutic procedures as well as a better understanding of the aetiology of the disease. The present study fulfilled some of these issues. The diagnosis of oral *Candida* carriage before the presentation of clinical symptoms allowed the possibility of (i) dental hygiene education for routine oral care, (ii) control of the spread of the colonization through the monitoring of colonization, and (iii) use of therapeutic approaches when appropriated. Finally, the main observation that may contribute to a better understanding of *Candida* oral carriage arose from the high frequency of polymicrobial cultures, which may represent an increased risk of infection to patients, requiring careful surveillance.

References

1. Cannon, R. D. & Chaffin, W. L. (1999) Oral colonization by *Candida albicans*. *Crit Rev Oral Biol Med* **10**, 359-383.
2. Cannon, R. D., Holmes, A. R., Mason, A. B., & Monk, B. C. (1995) Oral *Candida*: clearance, colonization, or candidiasis? *J Dent Res* **74**, 1152-1161.
3. Belazi, M., Velegraki, A., Fleva, A. *et al.* (2005) Candidal overgrowth in diabetic patients: potential predisposing factors. *Mycoses* **48**, 192-196.
4. Kleinegger, C. L., Lockhart, S. R., Vargas, K., & Soll, D. R. (1996) Frequency, intensity, species, and strains of oral Candida vary as a function of host age. *J Clin Microbiol* **34**, 2246-2254.

5. Meurman, J. H., Siikala, E., Richardson, M., & Rautemaa, R. (2007) Non-*Candida albicans Candida* yeasts of the oral cavity *Communicating Current Research and Educational Topics and Trends in Applied Microbiology.*, pp 719-731.
6. Zaremba, M. L., Daniluk, T., Rozkiewicz, D. *et al.* (2006) Incidence rate of *Candida* species in the oral cavity of middle-aged and elderly subjects. *Adv Med Sci* **51**, 233-236.
7. Henriques, M., Azeredo, J., & Oliveira, R. (2006) *Candida* species adhesion to oral epithelium: factors involved and experimental methodology used. *Crit Rev Microbiol* **32**, 217-226.
8. Akpan, A. & Morgan, R. (2002) Oral candidiasis. *Postgrad Med J* **78**, 455-459.
9. Farah, C. S., Ashman, R. B., & Challacombe, S. J. (2000) Oral candidosis. *Clin Dermatol* **18**, 553-562.
10. Odds, F. C. & Bernaerts, R. (1994) CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. *J Clin Microbiol* **32**, 1923-1929.
11. Kanbe, T., Horii, T., Arishima, T., Ozeki, M., & Kikuchi, A. (2002) PCR-based identification of pathogenic *Candida* species using primer mixes specific to *Candida* DNA topoisomerase II genes. *Yeast* **19**, 973-989.
12. Dahlen, G. (2006) Microbiological diagnostics in oral diseases. *Acta Odontol Scand* **64**, 164-168.
13. Willinger, B. & Manafi, M. (1999) Evaluation of CHROMagar Candida for rapid screening of clinical specimens for *Candida* species. *Mycoses* **42**, 61-65.
14. Yucesoy, M. & Marol, S. (2003) Performance of CHROMAGAR candida and BIGGY agar for identification of yeast species. *Ann Clin Microbiol Antimicrob* **2**, 8-15.
15. Murray, C. K., Beckius, M. L., Green, J. A., & Hospenthal, D. R. (2005) Use of chromogenic medium in the isolation of yeasts from clinical specimens. *J Med Microbiol* **54**, 981-985.
16. Saunte, D. M., Klingspor, L., Jalal, S., Arnau, J., & Arendrup, M. C. (2005) Four cases of *Candida albicans* infections with isolates developing pink colonies on CHROMagar Candida plates. *Mycoses* **48**, 378-381.
17. Daniluk, T., Tokajuk, G., Stokowska, W. *et al.* (2006) Occurrence rate of oral *Candida albicans* in denture wearer patients. *Adv Med Sci* **51**, 77-80.
18. de Resende, M. A., de Sousa, L. V., de Oliveira, R. C., Koga-Ito, C. Y., & Lyon, J. P. (2006) Prevalence and antifungal susceptibility of yeasts obtained from the oral cavity of elderly individuals. *Mycopathologia* **162**, 39-44.
19. Lockhart, S. R., Joly, S., Vargas, K., Swails-Wenger, J., Enger, L., & Soll, D. R. (1999) Natural defenses against Candida colonization breakdown in the oral cavities of the elderly. *J Dent Res* **78**, 857-868.
20. Webb, B. C., Thomas, C. J., Willcox, M. D. P., Harty, D. W. S., & Knox, K. W. (1998) *Candida* associated denture somastitis. Aetiology and management: a review. *Aust Dent J* **43**, 45-50.
21. Costa-de-Oliveira, S., Pina-Vaz, C., Mendonca, D., & Goncalves, R. A. (2008) A first Portuguese epidemiological survey of fungaemia in a university hospital. *Eur J Clin Microbiol Infect Dis* **27**, 365-374.
22. Goncalves, R. H., Miranda, E. T., Zaia, J. E., & Giannini, M. J. (2006) Species diversity of yeast in oral colonization of insulin-treated diabetes mellitus patients. *Mycopathologia* **162**, 83-89.
23. Ikebe, K., Morii, K., Matsuda, K., Hata, K., & Nokubi, T. (2006) Association of candidal activity with denture use and salivary flow in symptom-free adults over 60 years. *J Oral Rehabil.* **33**, 36-42.

24. Davies, A. N., Brailsford, S. R., & Beighton, D. (2006) Oral candidosis in patients with advanced cancer. *Oral Oncol.* **42**, 698-702.
25. Liguori, G., Lucariello, A., Colella, G., De Luca, A., & Marinelli, P. (2007) Rapid identification of *Candida* species in oral rinse solutions by PCR. *J Clin Pathol* **60**, 1035-1039.
26. Qi, Q. G., Hu, T., & Zhou, X. D. (2005) Frequency, species and molecular characterization of oral Candida in hosts of different age in China. *J Oral Pathol Med* **34**, 352-356.
27. Pfaller, M. A., Diekema, D. J., Gibbs, D. L. *et al.* (2007) Results from the ARTEMIS DISK Global Antifungal Surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J Clin Microbiol* **45**, 1735-1745.
28. Pfaller, M. A., Diekema, D. J., Gibbs, D. L. *et al.* (2008) Geographic and temporal trends in the isolation and antifungal susceptibility of *Candida parapsilosis*: a global assessment from the ARTEMIS DISK antifungal surveillance program, 2001-2005. *J Clin Microbiol* **46**, 842-849.
29. Pfaller, M. A., Diekema, D. J., Mendez, M. *et al.* (2006) *Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance Program. *J Clin Microbiol* **44**, 3551-3556.

CHAPTER 5

Concluding remarks and future perspectives

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Candida species live in complex and dynamic environments, being consequently exposed to external cues. In fact, to colonize so diverse niches such as the skin, gastrointestinal tract, or oral cavity, the cells need to adapt, for example, to different pH and oxygen levels, and microbial flora. This plasticity may involve the sensing and response to the multiple environmental inputs. It is currently accepted that one of the ways to coordinate the adaptation of the microbiological population is through cell to cell communication mediated by metabolites released by cells [1]. Therefore, one of the challenges in fundamental research is to identify the components that mediate cell to cell communication and to understand the nature, effect, and strength of the interactions on cell phenotype, to ultimately develop functional models (Fig.5-1) that may help to get a better understanding on pathogenesis [2].

In the last decade it was observed an increase in the "omics" approaches (Fig.5-1) in the *Candida* field, crucial to the elucidation of the fungal physiological responses to its environment. The genomic era started with *Candida albicans* [3] and *Candida glabrata* [4] genomes sequence, but only recently *Candida dubliniensis* [5], *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii*, and *Candida lusitaniae* [6], but not *Candida krusei* genome sequence were published. Additionally, transcriptomic studies, based on the dynamic expression of messenger ribonucleic acid (mRNA), have been used to identify genes relevant in a given physiological process, as dimorphism [7] or in particular niches such as biofilms (reviewed by Coenye [8]). Moreover, the proteomic studies, through the analysis of global patterns of protein expression, have been applied to the identification of key proteins related for example with cell adhesion, drug resistance, and virulence factors (reviewed by Thomas *et al.* [11]). Overall, the

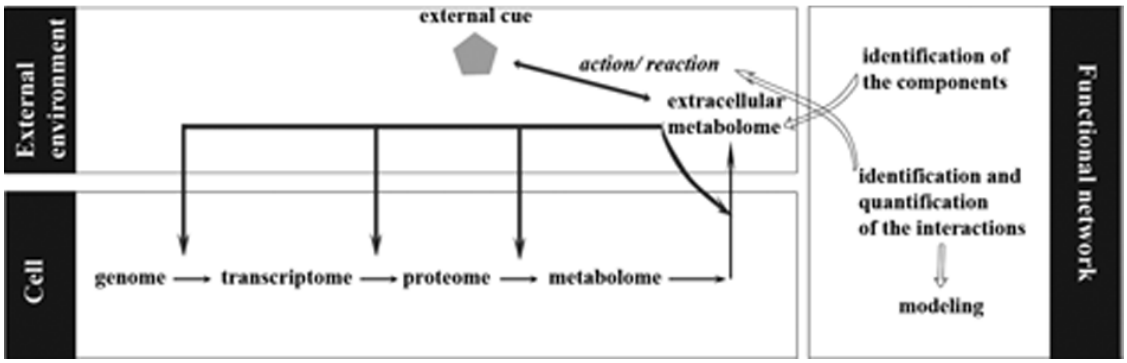

Fig.5-1. Schematic diagram of the functional principles and dynamics of the cellular systems adapted from [2, 9, 10].

combination of these approaches has been helpful to get a better understanding on *C. albicans* existence as a human commensal and pathogen.

However, the cells adjust their metabolism, and consequently their metabolites, according to the different physiological states [12]. The whole group of metabolites– the metabolome - link DNA, mRNA, proteins, complex interactions, and cellular pathways with environmental stimuli (Fig.5-1), thus being considered functional entities of the cells [13, 14]. Metabolomic (the quantification of the metabolome) or metabolic profiling (the quantification of a set of pre-defined metabolites belonging to a class of compounds or to members of particular pathways [12]) are unexploited topics in the *Candida* field. This can be inferred by the number of publications on the field: a Pubmed search using the terms "*Candida*" and "metabolite profile" or "metabolome" returns three and two publications, respectively, [http://www.ncbi.nlm.nih.gov/sites/pubmed (on 2010-07-06)], and these are not always representative of the subject. However, increased awareness is arising in the scientific community, with the "Biochemical Pathways" feature being recently added as a tool at the Candida Genome Database (CGD, http://www.candidagenome.org/) [15].

As pointed out in Chapter 1, one of the *Candida* extracellular metabolites that has been receiving particular attention in the last years is *E*,*E*-farnesol (farnesol). However, fungal extracellular compounds comprise so diverse molecules as volatiles, alcohol compounds, organic acids, extracellular enzymes, proteins [16], or DNA [17]. Due to the diversity of compounds and their dynamic regulation, the complete analysis of the metabolome is considered a difficult task [18]. Even though, it is possible to perform target analysis for the detection and quantification of a single or a small set of metabolites [12], thus achieving partial insights into the metabolome. In this thesis, to get insights into the extracellular milieu of *Candida* species, two different groups of extracellular molecules were evaluated: extracellular DNA (eDNA) (Chapter 2), and alcohol compounds (Chapter 3) (Fig.5-2 to Fig.5-5).

The interest on the study of eDNA, the first target molecule selected, was raised by evidences showing that: (i) eDNA accumulates into the extracellular medium in vitro and in vivo [17], (ii) eDNA can regulate the host immune response [19-21], and (iii) DNase I reduces biofilm biomass [22] (Chapter 1, Table 1-1). In fact, this molecule has not received considerable

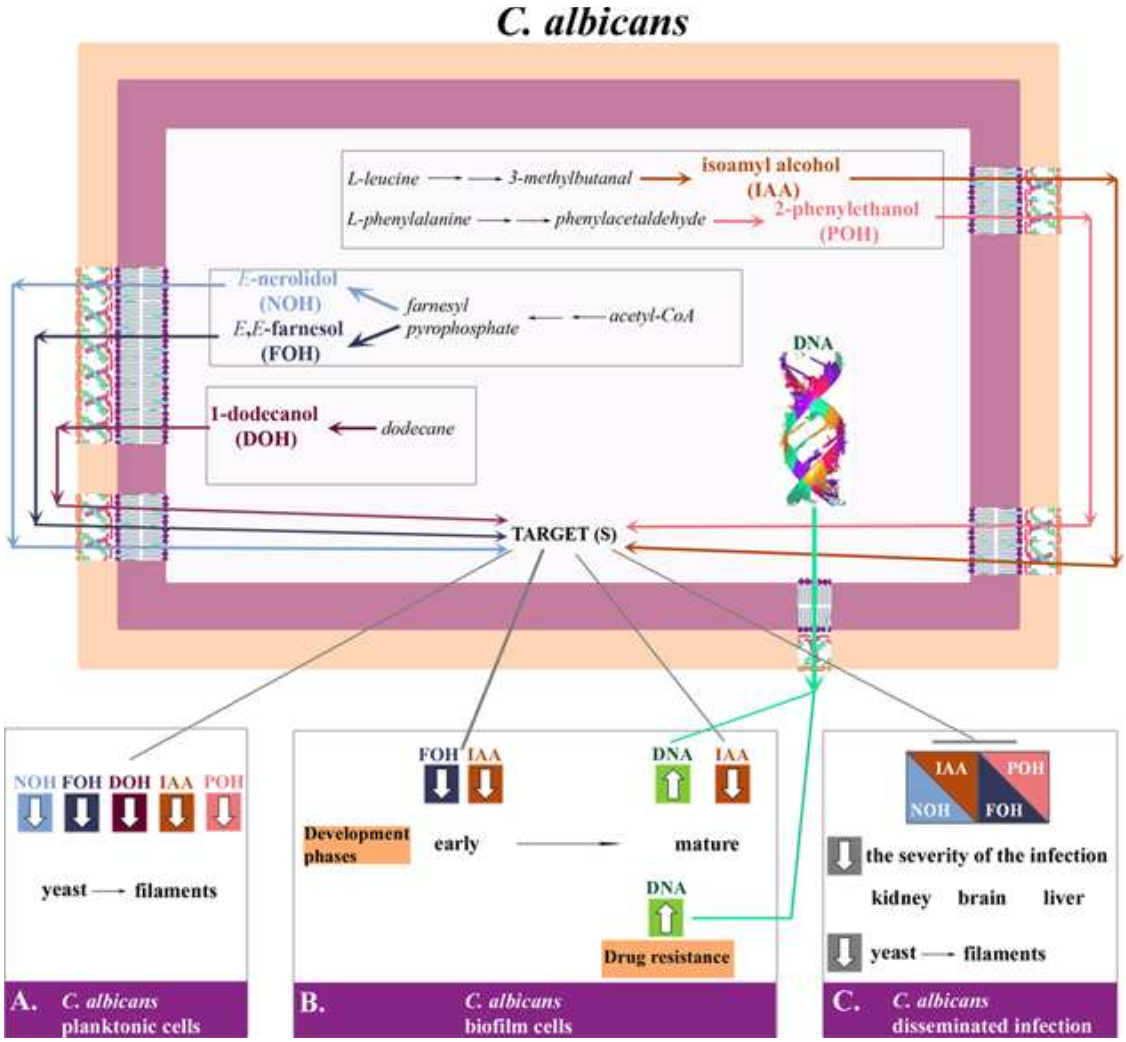

Fig.5-2. Overview of *C. albicans* extracellular compounds evaluated in this thesis and their effect in the organism's biology. *E,E*-farnesol (FOH) and 2-phenylethanol (POH) biosynthetic pathways as previously described by Hornby *et al.* [23] and Ghosh *et al.* [24], respectively. The represented isoamyl alcohol (IAA), *E*-nerolidol (NOH), and 1-dodecanol (DOH) biosynthetic pathways are hypothetical pathways predicted from the Candida Genome Database [15] and Zea *et al.* [25] and Walker [26] works. These alcohols and DNA are released into the extracellular medium. The effect of the individual addition of these alcohols was analyzed in terms of planktonic cells filamentation (***A***) and biofilm development (***B***). For the determination of the in vivo effect of alcohols against hematogenously disseminated candidiasis (***C***) the indicated alcohols were administered in a Cocktail solution. The role of eDNA on biofilm development and drug resistance was also evaluated (***B***). Large down arrows denote decrease and large up arrows denote increase in the biological effect.

considerable attention in the *Candida* biofilm field, and it has not been addressed, for example, whether it is a component of biofilm extracellular matrix (ECM) or not, or further exploited regarding its contribution to biofilm lifestyle.

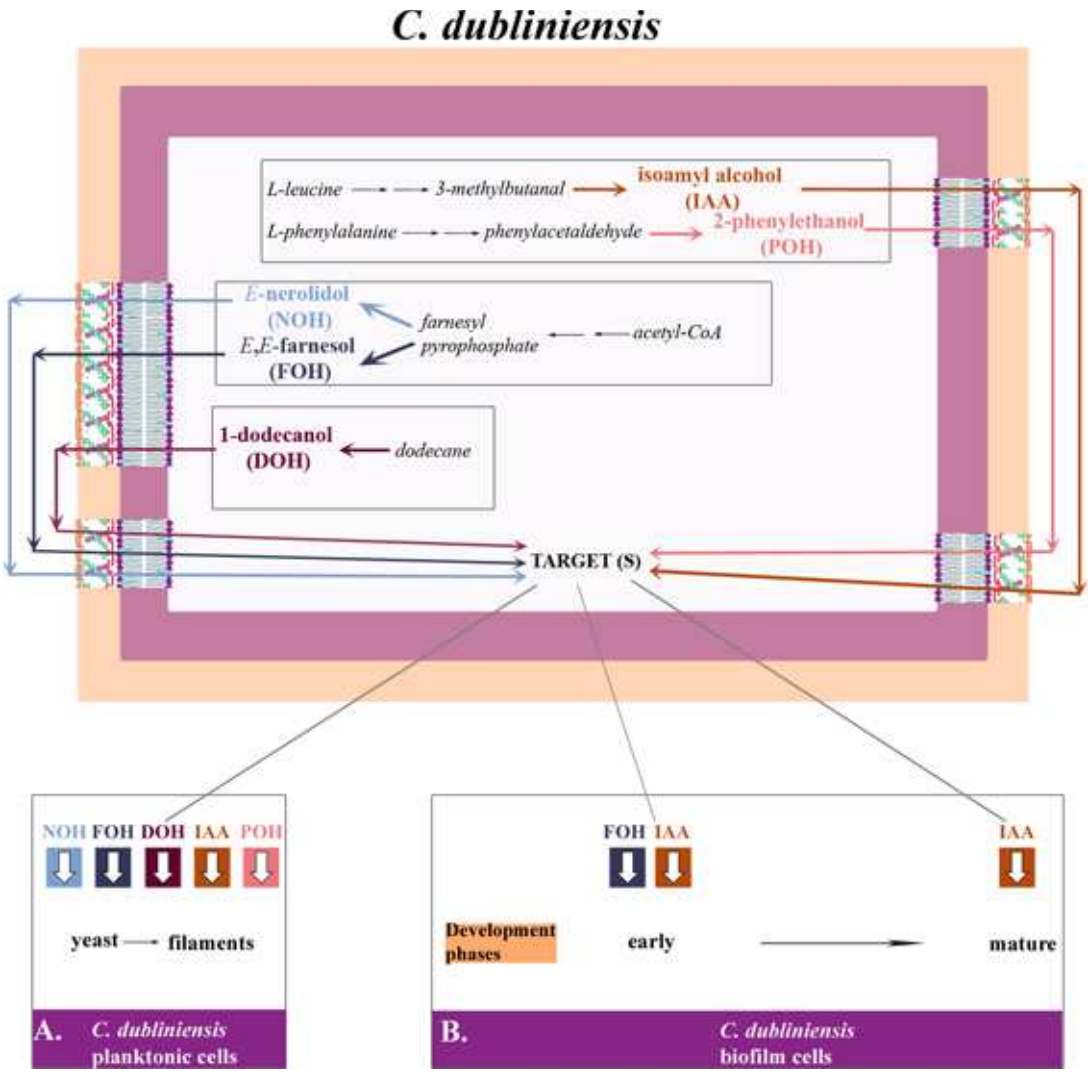

Fig.5-3. Overview of *C. dubliniensis* extracellular compounds evaluated in this thesis and their effect in the organism's biology. The represented isoamyl alcohol (IAA), 2-phenylethanol (POH), *E*-nerolidol (NOH), *E,E*-farnesol (FOH), and 1-dodecanol (DOH) biosynthetic pathways are hypothetical pathways predicted from the Candida Genome Database [15], Ghosh *et al.* [24], Zea *et al.* [25], Hornby *et al.* [23], and Walker [26] works. These alcohols are released into the extracellular medium. The effects of the individual addition of these alcohols was analyzed in terms of planktonic cells filamentation (***A***) and biofilm development (***B***). Large down arrows denote decrease and large up arrows denote increase in the biological effect.

Using a fluorometric method to quantify DNA in the ECM, it was shown that *C. albicans* mature biofilms are a reservoir of eDNA (Chapter 2.1). In parallel, another group [27] reported the presence of eDNA in *C. albicans* biofilms from a different strain, reinforcing our observations that eDNA is a component of *C. albicans* biofilms. But one of the points that is raising more controversy in the *Candida* scientific community relates to the origin of *C. albicans*

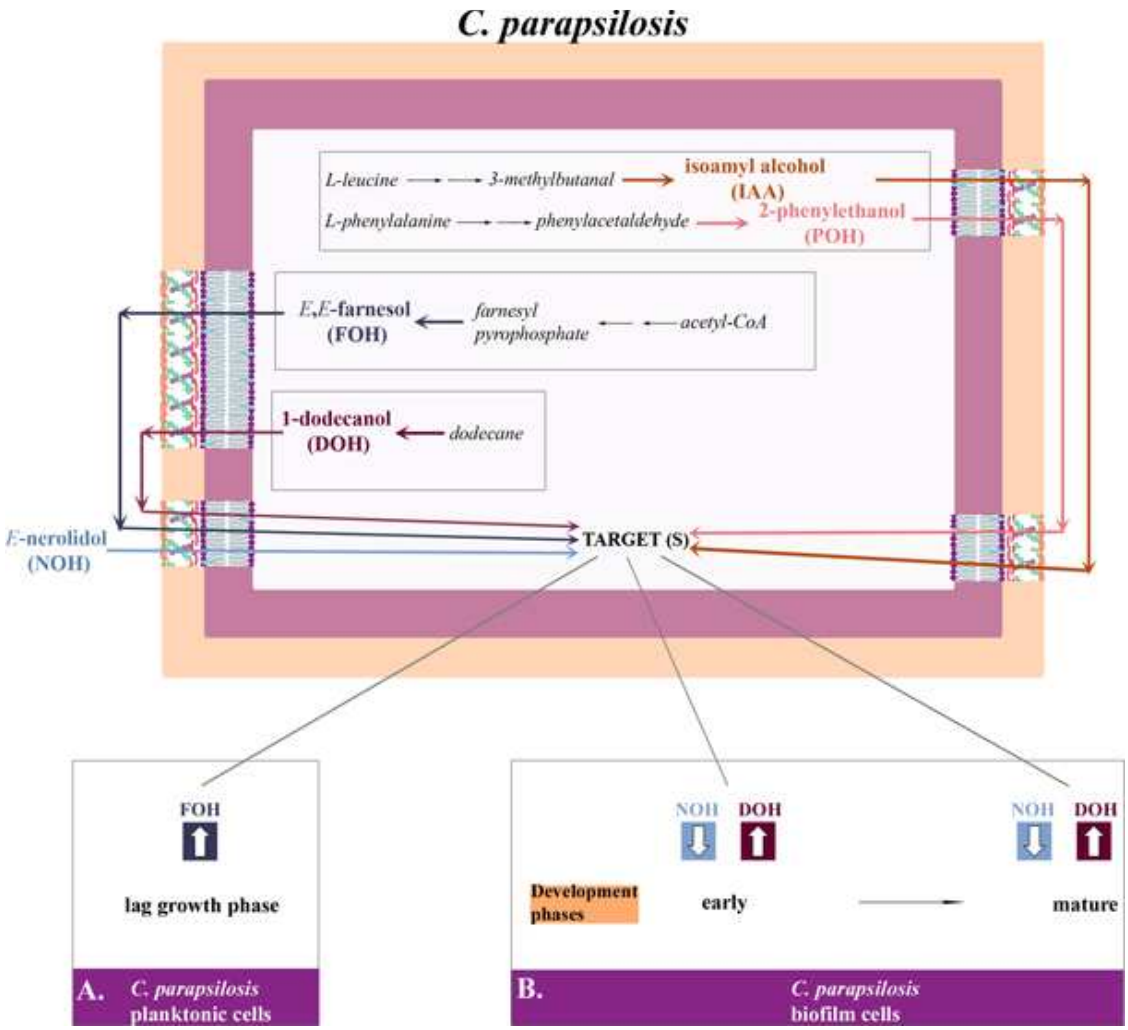

Fig.5-4. Overview of *C. parapsilosis* extracellular compounds evaluated in this thesis and their effect in the organism's biology. The represented isoamyl alcohol (IAA), 2-phenylethanol (POH), *E,E*-farnesol (FOH), and 1-dodecanol (DOH) biosynthetic pathways are hypothetical pathways predicted from the Candida Genome Database [15], Ghosh *et al.* [24], Zea *et al.* [25], Hornby *et al.* [23], and Walker [26] works. The effect of the individually addition of FOH was analyzed in terms of planktonic cells growth (***A***). The effects of the individual addition of *E*-nerolidol (NOH), IAA, POH, FOH, and DOH were analyzed in terms of biofilm development (***B***). Large down arrows denote decrease and large up arrows denote increase in the biological effect.

eDNA. Accumulated evidences in the bacterial field suggest that eDNA may be released released through quorum sensing and specific secretion mechanisms, and cell lysis (reviewed in [28, 29]). The evaluation of eDNA content of biofilms formed by a *C. albicans* mutant strain, known to be non-responsive to farnesol [27], suggests that the release of eDNA is not dependent on quorum sensing mediated by farnesol. However, it may not be disregarded that other extracellular alcohols released by *C. albicans* (Chapter 3.2) may regulate eDNA release.

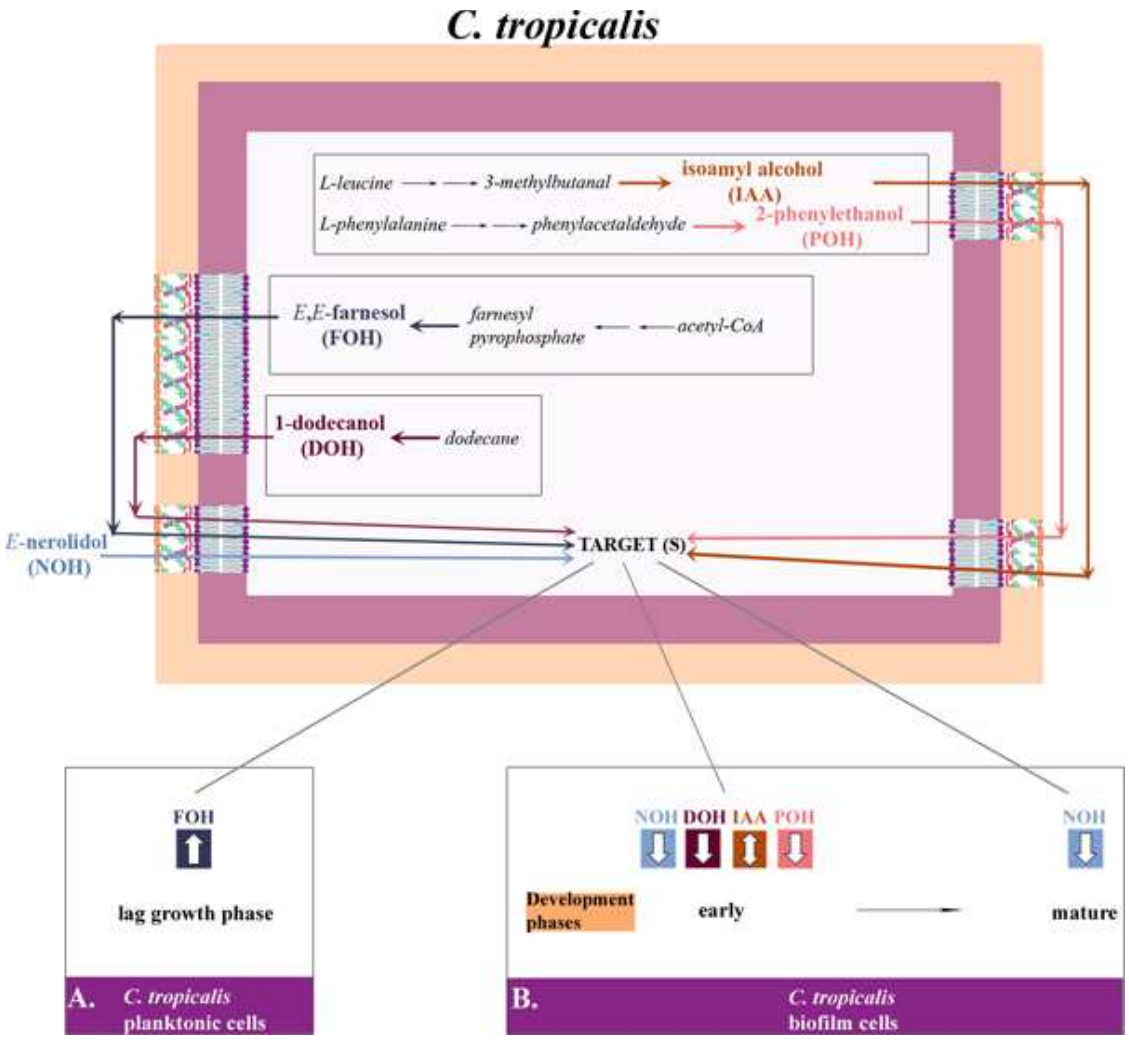

Fig.5-5. Overview of *C. tropicalis* extracellular compounds evaluated in this thesis and their effect in the organism's biology. The represented isoamyl alcohol (IAA), 2-phenylethanol (POH), *E,E*-farnesol (FOH), and 1-dodecanol (DOH) biosynthetic pathways are hypothetical pathways predicted from the Candida Genome Database [15], Ghosh *et al.* [24], Zea *et al.* [25], Hornby *et al.* [23], and Walker [26] works. These alcohols are released into the extracellular medium. The effect of the individual addition of FOH was analyzed in terms of planktonic cells growth (***A***). The effects of the individual addition of *E*-nerolidol (NOH), IAA, POH, FOH, and DOH were analyzed in terms of biofilm development (***B***). Large down arrows denote decrease, large up arrows denote increase in the biological effect. Large up and down arrow denote a different behavior in the different parameters evaluated concerning the same biological effect.

In fact, in the *Candida* field, the most accepted hyphothesis is that eDNA is a by-product derived from cell lysis within the biofilm (personnal communications on internacional conferences). Our preliminary studies on the evaluation of the eDNA electrophoretic mobility in conventional agarose gels containing ethidium bromide showed that eDNA is not similar to genomic DNA, exhibiting a smear profile (ranging approximately from 5000 to 75 bp) (unpublished data). This suggests that *C. albicans* eDNA is derived from cell death [30]. However, research is still

needed: (i) to establish a possible correlation between cell death (necrosis and/or apoptosis), eDNA release and biofilm formation, and (ii) to exploit other origin(s) of eDNA.

In addition, the role of eDNA was addressed in terms of biofilm development (Chapter 2.1, Fig.5-2) and antifungal susceptibility (Chapter 2.2, Fig.5-2). Based on the degradation of eDNA and addition of exogenous DNA to biofilms at different stages of biofilm development, it was possible to define for the first time the relevance of eDNA during *C. albicans* biofilm development, showing that it contributes to the maintenance and stability of mature biofilms, but not to their establishment (Chapter 2.1).

Although it is recognized that biofilm stability/dispersion is a crucial step in the biofilm lifecycle (Chapter 1, Fig.1-2), only recently the phenomenon of *C. albicans* biofilm dispersion began to be detailed. In a pioneer study, Uppuluri *et al.* [31] showed that the peak of cell dispersal occurs at the intermediate stage of biofilm development, being this process controlled by environmental and molecular factors. Additionally, the dispersed cells sum properties indicating that they are more virulent than the planktonic counterparts [31]. In light of these findings, it may be interesting to continue our studies by evaluating the phenotypic properties of the cells that are held together by DNA, and thus dispersed in the presence of DNase, in terms of morphology, adherence, ability to form biofilm, and in vivo virulence.

One of the aspects not focused on our studies (Chapter 2.1) was the existence and/or role of eDNA derived from biofilm cells but not embedded within the ECM. In fact, it is possible that a portion of the eDNA released by biofilm cells is not trapped by the ECM, being released to the surrounding environment. It may be interesting to address: (i) how circulating eDNA derived from biofilm cells circulates, since it is known that proteins are able to bind DNA [32], and (ii) the role of biofilm-derived eDNA in the modulation of the host immune system. A step forward in the research on eDNA in *Candida* species, is the evaluation of the potential use of eDNA as a marker for the diagnostic and prognostic of *Candida* infections, similarly to the observed for β-glucans [33].

In addition, data presented in Chapter 2.2 suggest a novel role for eDNA in *C. albicans* biofilms, as a regulator of antifungal susceptibility (Fig.5-2). The studies performed show that DNase increases the effectiveness of amphotericin B and caspofungin against *C. albicans* biofilms (Chapter 2.2). Notably, in the last years the combined use of drugs [34] or drugs with other agents, such as enzymes [35], has received considerable attention. ECM degrading

enzymes present a broad spectrum activity, it is unlikely that they induce antimicrobial resistance, and their current use as therapeutic agents (e.g. DNase is already used for cystic fibrosis treatment), suggests their tolerance and activity in a clinical setting. However, it may not be ignored that enzyme-based therapies are expensive and may increase the risk of distal sites colonization due to biofilm cells dispersion [35]. Even though, our studies open the possibility of treating biofilm device related *Candida* infections, with lower antifungal levels, which may be beneficial considering the toxicity of polyene drugs [36] and the emergence of *Candida* echinocandin resistance [37]. Nevertheless, the potential relevance of these findings (Chapter 2.2), requires further experiments in order: (i) to understand whether the reduced levels of mitochondrial cell activity represent an effective reduction in metabolism or are resultant of cell dispersion, (ii) to determine the fractional inhibitory concentration index as a measure of synergy between DNase and these antifungal agents, and (iii) to evaluate more drugs from each antifungal class. Furthermore, for the consideration of DNase as an anti-candidal enzyme, it is necessary to evaluate eDNA presence and function on an additional number of *C. albicans* strains and other *Candida* species, including clinical samples such as those identified in this thesis (Chapter 4).

In fact, the potential therapeutic use of ECM degrading enzymes is not a new issue in the *Candida* field, since the enhanced efficacy of the combined use of β-1,3-glucanase and antifungal agents in the eradication of *C. albicans* biofilms has been previously shown by Nett el al. [38]. This corroborates our observations (Chapter 2) that agents that target processes affecting the biofilm structural integrity may have potential use as therapeutic adjuvants in biofilm treatment, at least as part of antifungal lock therapy of devices.

Overall, the results of the set of experiments reported in Chapter 2 show that eDNA is a component of *C. albicans* biofilm ECM that contributes to biofilm integrity and antifungal resistance (Fig.5-2). Introducing a new line of research, these studies expanded the current knowledge on *C. albicans* biofilm ECM.

The second set of molecules selected were extracellular alcohols (Chapter 3, summarized on Fig.5-2 to Fig.5-5). Extracellular alcohols were pointed as targets for our research due to: (i) the discovery of farnesol as a quorum sensing molecule in *C. albicans*, and (ii) sparse studies suggesting that yeast cells release autoregulatory molecules into the extracellular medium

(Chapter 1, Table 1-1). As observed in other *Candida* research areas, the investigation on Non-*Candida albicans Candida* (NCAC) species is scarce, and for example not much is known on the role of farnesol on these species. In addition, the identification of extracellular molecules released by *C. albicans*, and consequently NCAC species, is dependent on appropriate analytical methods. Overcoming this constraint will open the possibility of exploiting the interaction of the identified molecules with virulence traits of *Candida* species.

Previous investigations on the effect of farnesol on NCAC species planktonic cells suggested a species dependent response: (i) in *C. dubliniensis*, morphology control (Chapter 3.2, Fig.5-3, [39]), and (ii) in *C. parapsilosis* growth regulation [40]. Having found in our experiments the same farnesol induced effect in *C. parapsilosis* we perused the effect of the addition of exogenous farnesol in other NCAC species planktonic cells (Chapter 3.1). Using a set of methodologies that allow morphology, growth, viability, and cell cycle monitoring, it was found that farnesol (≥ 50 μM) affects *C. glabrata*, *C. krusei*, and *C. tropicalis* (Fig.5-4) growth phase, with distinct effects in cell survival and cell cycle, without changes in cell morphology (Chapter 3.1). Notably, very recently, an independent group reported the growth inhibitory effect of farnesol on NCAC species [41], although the cytotoxic process was not assessed, contrarily to the evaluation performed in our study (Chapter 3.1).

Despite the considerable insights into the role of farnesol in NCAC species two main questions remain unanswered. First, farnesol receptors or farnesol binding proteins have not been identified so far [42]. However, in some cell types, receptors regulated by farnesol and its derivates were identified, namely, farnesoid X receptor and peroxisome proliferator-activated receptors [43]. Due to the similarities shared between animals and fungi [44], the existence of these receptors in *Candida* species, as well as the hypothesis of these proteins being farnesol receptors should be addressed. Second, the potential farnesol targets inside NCAC species cells are not fully elucidated. From the studies performed in *C. albicans* there are evidences supporting the involvement of the cAMP signaling pathway in the farnesol mediated response [45]. In parallel, this pathway has been shown to regulate *C. albicans* cell growth, and cell cycle [46]. With these studies in the backdrop, dibutyryl-cyclic adenosine monophosphate (dibutyryl-cAMP) (10 mM) was added into NCAC species culture medium following the addition of farnesol (150 μM), and cell growth and morphology were monitored. *C. albicans* was used as a positive control. Unfortunately, we were not successful in the validation of the assay, since *C. albicans*

farnesol mediated filamentation inhibition was not reversed by dibutyryl-cAMP, as previously described [45]. However, as noted in the beginning of this chapter, only recently the NCAC species genome sequences were released. From now on, it will be possible to develop for example oligonucleotide microarrays, tool required for the analysis of global pattern gene expression of NCAC species, for example, under the exposure to farnesol. In between, based on the insights given by Rossignol *et al.* [40] on the effect of farnesol in *C. parapsilosis*, other targets, such as lipid metabolism, ribosome biogenesis, and amino acid biosynthesis may be further evaluated in *C. glabrata*, *C. krusei*, and *C. tropicalis*.

As shown in Chapters 3.2 and 3.3 farnesol production into the extracellular medium is a common feature of *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* species planktonic cells. Moreover, we were also able to identify farnesol in *C. krusei* ATCC 6258 and *C. glabrata* ATCC 2001 supernatants (unpublished data). Although the regulation of farnesol production by *Candida* species is still under debate, the farnesol levels considered physiologically relevant are below 50 μM [42]. It should be noted that physiological farnesol levels do not affect *C. glabrata*, *C. krusei*, and *C. tropicalis* growth (Chapter 3.1), but they regulate *C. albicans* and *C. dubliniensis* morphology (Chapter 3.2). This suggests that farnesol acts more likely as a quorum sensing molecule in *C. albicans* and *C. dubliniensis*, regulating a specific virulence trait of these *Candida* species [47, 48], than in the other ones.

In addition, ongoing research in our lab suggests that *C. albicans* releases into the extracellular medium other molecules that may interfere with NCAC species physiology. Specifically, *C. albicans* supernatant: (i) does not exhibit major effects on *C. glabrata* and *C. dubliniensis* growth, (ii) promotes *C. krusei* and *C. parapsilosis* cell growth, but (iii) has a growth inhibitory effect on *C. tropicalis*. It is interestingly to note that the identification of *Candida* species in the oral cavity of patients attending a dental clinic (Chapter 4) revealed that the most common polymicrobial association was between *C. albicans* and *C. glabrata*, although *C. parapsilosis* and *C. tropicalis* were also isolated in combination with *C. albicans*. In addition, *C. albicans* and *C. dubliniensis* supernatants are able to regulate their own morphology (Chapter 3.2). These findings prompted our research to the characterization of the molecules present in the extracellular medium and the evaluation of their effect.

A number of techniques have been employed for fungal metabolites profiling including paper chromatography, thin layer chromatography, capillary electrophoresis, and, the mainly

applied, high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC–MS) [49]. Although chromatographic methods are very sensitive and selective their major disadvantage relates with the sample preparation methods, traditionally time consuming, expensive, and requiring a large volume of hazardous organic solvents [50]. However, in the beginning of the last decade solid-phase microextraction (SPME) technology was introduced to overcome some of these difficulties. SPME is a technique based in an equilibrium partition of the compounds between the sample matrix and the extracting phase, a polymer-coated fused fiber that can be directly coupled with HPLC or GC-MS [51]. One of the drawbacks of this technique for a full characterization of the metabolome is the fact that the compounds extracted are dependent on the SPME fiber coating and thickness [52]. In fact, the Cross-linked Carbowax-divinylbenzene coating fibre used in the studies presented in this thesis (Chapters 3.2 and 3.3) is recommended for small and polar molecules (molecular weight between 40 and 275), determining the target analysis of specific compounds.

Using headspace-SPME (HS-SPME) coupled to GC-MS, it was established, for the first time, the profile of multiple extracellular alcohols produced during *C. albicans* (Chapter 3.2, Fig.5-2), *C. dubliniensis* (Chapter 3.2, Fig.5-3), *C. parapsilosis* (Chapter 3.3, Fig.5-4), and *C. tropicalis* (Chapter 3.3, Fig.5-5) in vitro growth. In an unified and detailed study along time (from 24 to 96 h), a group of molecules sharing an alcohol group- isoamyl alcohol, 2-phenylethanol (phenylethanol), 1-dodecanol (dodecanol), *E*-nerolidol (nerolidol), and farnesol- were identified and quantified in planktonic and biofilm supernatants of *C. albicans* (Chapter 3.2, Fig.5-2) and *C. dubliniensis* (Chapter 3.2, Fig.5-3). Although alcohol secretion profiles were species, culture mode, and growth time specific, it was not possible to establish a biofilm fingerprint concerning these alcohols identity (Chapter 3.2. Therefore, as an initial approach to get insights into extracellular alcohols produced by other *Candida* species, only planktonic supernatants were analyzed. Specifically, *C. parapsilosis* (Chapter 3.3, Fig.5-4), *C. tropicalis* (Chapter 3.3, Fig.5-5), *C. glabrata,* and *C. krusei* (unpublished data) 24-h supernatants contained not only farnesol, as described earlier in this chapter, but also isoamyl alcohol, phenylethanol, and dodecanol. Nerolidol was only detected in *C. glabrata* and *C. krusei* supernatants. The continuous need of improvement of the analytical methods for the analysis of *Candida* extracellular alcohols is evidenced by a recent study describing the development of an ultra high performance liquid chromatography tandem mass spectrometry method for the analysis of extracellular alcohols

[53]. However, the method was only optimized for the identification and quantification of farnesol and tyrosol, in contrast to HS-SPME GC-MS methodology described in this thesis that allows the simultaneous analysis of several alcohols.

It has been suggested that the majority of the molecules secreted by microorganisms are able to modulate physiological functions, although the observed effects are not always derived from a quorum sensing circuit but many times resultant from processes of compound metabolisation or detoxification [54]. It is interesting to note that the identified molecules (Chapters 3.2 and 3.3) have a volatile nature, which may help the signal diffusion, communication between cells, and consequently the regulation of virulence factors. In a recent publication, Diggle *et al.* [55] proposed a classification to distinguish the types of communication between cells. This classification differentiates signal, cue, and coercion depending on the consequences to the sender and the responder cell. Specifically, a molecule should be classified as a signal when it alters the behavior of a receiver cell and this response benefits the producer cell. This concept suggests that the signal evolved owing to the response it elicits. A molecule is considered as a cue if its effect is beneficial for the receiver but it does not benefit the sender cell, thus it has not evolved owing to its effect on the receiver cell. Additionally, a molecule may coerce a receiver cell into an action that does not benefit this cell, although it benefits the sender. So, one of the challenges is to determine the effect to the sender and to the receiver cells, and thus infer the costs and benefits of communication between cells. In this sense, to get insights into the interaction of the identified molecules (Chapters 3.2 and 3.3) with virulence traits of *Candida* species, the effect of the individual addition of commercial formulations of the identified alcohols, at physiological and/or supraphysiological concentrations (using as reference the *C. albicans* and *C. dubliniensis* produced levels – Chapter 3.2), was examined in terms of *Candida* species morphogenesis, growth regulation (Chapter 3.2, Fig.5-2 and Fig.5-3), biofilm formation (Chapter 3.3, Fig.5-2 to Fig.5-5), and in the progression of disseminated candidiasis (Chapter 3.4, Fig.5-2).

First, from our studies on the effect of *C. albicans* and *C. dubliniensis* extracellular alcohols in their planktonic cells morphology, it was found that under filamentation inducing conditions, these compounds inhibit the yeast to filamentous form conversion (Fig.5-2 and Fig.5-3) without significant growth constraints (Chapter 3.2). These findings added isoamyl alcohol, phenylethanol, dodecanol, and nerolidol as *Candida* species autoregulatory substances

involved in the morphogenesis regulation. However, in *C. albicans* the intracellular targets of isoamyl alcohol, phenylethanol, and nerolidol are not known, and in a first approach, the evaluation of the expression of genes involved in key pathways of morphogenesis [56] may be helpful. However, the knowledge on *C. dubliniensis* filamentation regulation by extracellular alcohols is even scarcer than in *C. albicans*. Thus, it may be particularly interesting to elucidate these pathways, since it has been suggested that the dimorphism regulation may differ between *C. albicans* and *C. dubliniensis* [47].

In addition, morphogenesis regulation is not considered a virulence trait of other *Candida* species such as *C. parapsilosis, C. glabrata*, *C. krusei*, and *C. tropicalis* [57], and as suggested by data in Chapter 3.1, extracellular alcohols may not interfere with it. Although the effect of the exogenous addition of isoamyl alcohol, phenylethanol, dodecanol, and nerolidol has not been tested in these NCAC species in terms of morphology, our preliminary studies suggest that these compounds do not interfere with their growth (unpublished data).

Second, by screening the effect of exogenously added isoamyl alcohol, phenylethanol, dodecanol, nerolidol, and farnesol on biofilms (Chapter 3.3) it was shown, for the first time, that other alcohol compounds, besides farnesol, regulate *C. albicans* (Fig.5-2), *C. dubliniensis* (Fig.5-3), *C. parapsilosis* (Fig.5-4), and *C. tropicalis* (Fig.5-5) biofilm development. Specifically, (i) isoamyl alcohol elicited anti-biofilm activity against *C. albicans* and *C. dubliniensis*, and a heterogeneous activity against *C. tropicalis* biofilm cells, (ii) phenylethanol showed anti-biofilm activity against *C. tropicalis*, (iii) dodecanol elicited a pro-biofilm activity in *C. parapsilosis* but anti-biofilm activity in *C. tropicalis*, (iv) nerolidol showed an anti-biofilm activity against *C. parapsilosis* and *C. tropicalis*, and (v) farnesol reduced *C. albicans* and *C. dubliniensis* biofilm (Chapter 3.3, Fig.5-2 to Fig.5-5). It should be noted that we attempted to test the effect of these alcohols in *C. glabrata* and *C. krusei* biofilm development. However, the standard methods used to study biofilm cells mitochondrial activity ([2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide]- XTT) and biofilm biomass (crystal violet assay) (Chapter 3.3), exhibited poor sensitivity and reproducibility for *C. glabrata* and *C. krusei*. In fact, other authors warned for these difficulties, specifically with the use of XTT on NCAC species [58], pointing out to the need for optimization of the existing methods or the development of new robust methods to study *C. glabrata* and *C. krusei* biofilms. Concerning the effect of the evaluated alcohols on biofilm development (Chapter 3.3), further studies are still required to evaluate: (i) the

production of the different extracellular alcohols by the NCAC species biofilm cells, (ii) the impact of increasing the range of concentrations tested when these alcohols showed an effect, and (iii) other parameters such as biofilm cells morphology, and ECM composition, including eDNA. In addition, it would be interesting to elucidate how the cells in different regions of the biofilm respond to the addition of the alcohols. This can now be accomplished by cryosectioning the biofilms and using capture microdissection microscopy coupled with multiplex quantitative real-time reverse transcriptase polymerase chain reaction [59].

Third, as these alcohols were not individually identified in the culture supernatants (Chapters 3.2 and 3.3), they may not act isolate but rather as part of a network. To clarify the biological relevance of these alcohols, *C. albicans* and *C. dubliniensis* 96 h planktonic and biofilm supernatants were mimicked using Cocktail solutions containing commercial formulations of isoamyl alcohol, phenylethanol, nerolidol, and farnesol at the concentrations determined (Chapter 3.2). In general, the in vitro effect of the Cocktail mixtures, on *C. albicans* and *C. dubliniensis* filamentation inhibition, was similar to that of the corresponding supernatants (Chapter 3.2). The exception was the Cocktail solution mimicking *C. dubliniensis* planktonic supernatant (Chapter 3.2), suggesting that other morpho-regulatory molecules may be present in these fractions. Following this line, on one of our ongoing studies, the complete profile of compounds secreted by *C. albicans* and *C. dubliniensis* is being determined and the effect of the molecules unraveled (unpublished data). For example, it was found that menthol is present in both *C. albicans* and *C. dubliniensis* planktonic and biofilm supernatants. The addition of the corresponding commercial standard to the culture medium at physiological levels (< 1 μg/l) inhibits these *Candida* species growth.

Fourth, an indication of the in vivo relevance of these alcohols (Chapter 3.2) was shown during infection in a mouse model of disseminated *C. albicans* candidiasis (Chapter 3.4, Fig.5-2). The exogenous administration of the *C. albicans* Cocktail solution displayed the capacity to modify the course of the infection and decrease its severity, by increasing mice survival, decreasing organ fungal burden, and inhibiting filamentation. It is still to explore how the Cocktail solution interferes with the host immune system, thus modulating the disease progression. This could be achieved by profiling cytokines with systems such as the Bio-Plex®, that allow the simultaneously quantification of up to 23 mouse cytokines. In addition, it would be interesting to elucidate the contribution of each individual alcohol in the progression of the

disseminated disease. Moreover, evidences in literature suggest that the role of farnesol in vivo depends on the disease model [60, 61]. In light of our findings (Chapter 3) it would be of interest: (i) to test the effect of the Cocktail solution in the recently developed animal biofilm models [62, 63], and (ii) to ascertain the in vivo distribution and metabolism of these compounds, which can be achieved by the use of emerging imaging techniques such as mass spectrometry imaging [64].

Although these studies (Chapter 3) began to dissect the regulation of *Candida* species biology through autoregulatory molecules, there are additional points that could be addressed throughout, such as: (i) the elucidation of the metabolic pathways underlying the production of these compounds, for example by the use of ^{13}C nuclear magnetic resonance spectroscopy, as previously performed for *Saccharomyces cerevisiae* [65], (ii) the study of the physiological conditions that regulate the production of the these alcohols in the planktonic and biofilm lifestyle, (iii) the assessment of the externally added compounds metabolization by the cells, (iv) the evaluation of the effect of the alcohols on other virulence attributes, such as adhesins, proteolytic and hydrolytic enzymes secretion [47, 48, 66, 67], and (v) the increase in the number of strains of each *Candida* species evaluated.

The results of this set of experiments (Chapter 3) report that other alcohol molecules, besides farnesol are produced by *Candida* species, and that these molecules are able to modulate filamentation, biofilm development, and disease pathogenesis, depending on the alcohol and on the *Candida* species. In short term, these findings increased the current understanding on the regulation of physiology and virulence traits of *Candida* species through autoregulatory molecules.

Remarkably, it may be noted that it is difficult to establish costs and benefits of communication between cells when several parameters are being evaluated (Chapters 3.2 and 3.3). In addition, studies such as those presented in Chapter 4, shed some light into the complexity of the natural environments that may influence the role of the molecules in the regulation of *Candida* phenotype. Actually, in a natural environment, the signal molecules may be washed out being the concentration of the compounds a balance between their production, degradation or half-life time [68]. In addition, in these systems, such as the oral cavity (evaluated in Chapter 4), other components may interfere with the balance of extracellular molecules, such as host components and oral bacteria, that make part of oral *C. albicans*

biofilms [69]. Curiously, it was recently found that the oral bacterium *Streptococcus mutans* produces a molecule similar to farnesol, *E*-2-decenoic acid, that inhibits *C. albicans* filamentation. So, one of the major challenges in the future is to be able to distinguish the differential production of molecules by the different species in different environments, in order to establish the comprehensive and accurate dynamic changes of molecules within a cell, which can only be achieved by the use of modeling tools (Fig.5-1) [13].

In summary, the information resulting from this thesis contributes to a better understanding of the extracellular milieu, not only of *C. albicans* but also of NCAC species. In short term, we increased our knowledge on specific components of *Candida* species extracellular medium- eDNA and alcohol compounds- and on their interaction with *Candida* species phenotype. In long term, the insights achieved can open the doors to other investigations that can lead to the development of treatment and/ or diagnostic strategies to combat disseminated candidiasis.

REFERENCES

1. Zhang, L. H. & Dong, Y. H. (2004) Quorum sensing and signal interference: diverse implications. *Mol Microbiol* **53**, 1563-1571.
2. Sauer, U., Heinemann, M., & Zamboni, N. (2007) Genetics. Getting closer to the whole picture. *Science* **316**, 550-551.
3. Jones, T., Federspiel, N. A., Chibana, H. *et al.* (2004) The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci USA* **101**, 7329-7334.
4. Dujon, B., Sherman, D., Fischer, G. *et al.* (2004) Genome evolution in yeasts. *Nature* **430**, 35-44.
5. Jackson, A. P., Gamble, J. A., Yeomans, T. *et al.* (2009) Comparative genomics of the fungal pathogens *Candida dubliniensis* and *C. albicans*. *Genome Res* **19**, 2231-2244.
6. Butler, G., Rasmussen, M. D., Lin, M. F. *et al.* (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459**, 657-662.
7. Nantel, A., Dignard, D., Bachewich, C. *et al.* (2002) Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* **13**, 3452-3465.
8. Coenye, T. (2010) Response of sessile cells to stress: from changes in gene expression to phenotypic adaptation. *FEMS Immunol. Med Microbiol* **in press**.
9. Smedsgaard, J. & Nielsen, J. (2005) Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. *J Exp Bot* **56**, 273-286.

10. Frutos, R., Viari, A., Vachiery, N., Boyer, F., Lefrancois, T., & Martinez, D. (2008) Emergence and potential of high-throughput and integrative approaches in pathology. *Ann NY Acad Sci* **1149**, 62-65.
11. Thomas, D. P., Pitarch, A., Monteoliva, L., Gil, C., & Lopez-Ribot, J. L. (2006) Proteomics to study *Candida albicans* biology and pathogenicity. *Infect Disord Drug Targets* **6**, 335-341.
12. Oldiges, M., Lutz, S., Pflug, S., Schroer, K., Stein, N., & Wiendahl, C. (2007) Metabolomics: current state and evolving methodologies and tools. *Appl Microbiol Biotechnol* **76**, 495-511.
13. Zhang, W., Li, F., & Nie, L. (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology* **156**, 287-301.
14. Jewett, M. C., Hofmann, G., & Nielsen, J. (2006) Fungal metabolite analysis in genomics and phenomics. *Curr Opin Biotechnol* **17**, 191-197.
15. Skrzypek, M. S., Arnaud, M. B., Costanzo, M. C. *et al.* (2010) New tools at the Candida Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res* **38**, D428-D432.
16. Frisvad, J. C., Larsen, T. O., de, V. R. *et al.* (2007) Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. *Stud Mycol* **59**, 31-37.
17. Kasai, M., Francesconi, A., Petraitiene, R. *et al.* (2006) Use of quantitative real-time PCR to study the kinetics of extracellular DNA released from *Candida albicans*, with implications for diagnosis of invasive candidiasis. *J Clin Microbiol* **44**, 143-150.
18. Villas-Boas, S. G., Rasmussen, S., & Lane, G. A. (2005) Metabolomics or metabolite profiles? *Trends Biotechnol* **23**, 385-386.
19. Yordanov, M., Dimitrova, P., Danova, S., & Ivanovska, N. (2005) *Candida albicans* double-stranded DNA can participate in the host defense against disseminated candidiasis. *Microbes Infect* **7**, 178-186.
20. Dimitrova, P., Yordanov, M., Danova, S., & Ivanovska, N. (2008) Enhanced resistance against systemic *Candida albicans* infection in mice treated with *C. albicans* DNA. *FEMS Immunol Med Microbiol* **53**, 231-236.
21. Miyazato, A., Nakamura, K., Yamamoto, N. *et al.* (2009) Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*. *Infect Immun* **77**, 3056-3064.
22. Al-Fattani, M. A. & Douglas, L. J. (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* **55**, 999-1008.
23. Hornby, J. M., Kebaara, B. W., & Nickerson, K. W. (2003) Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. *Antimicrob Agents Chemother* **47**, 2366-2369.
24. Ghosh, S., Kebaara, B. W., Atkin, A. L., & Nickerson, K. W. (2008) Regulation of aromatic alcohol production in *Candida albicans*. *Appl Environ Microbiol* **74**, 7211-7218.
25. Zea, L., Moreno, J., Ortega, J. M., & Medina, M. (1995) Content of free terpenic compounds in cells and musts during vinification with three *Saccharomyces cerevisiae* races. *J Agric Food Chem* **43**, 1110-1114.
26. Walker, G. M. (1998) *Yeast physiology and biotechnology*. London: John Wiley & Sons (ed)
27. Paramonova, E., Krom, B. P., van der Mei, H. C., Busscher, H. J., & Sharma, P. K. (2009) Hyphal content determines the compression strength of *Candida albicans* biofilms. *Microbiology* **155**, 1997-2003.

28. Draghi, J. A. & Turner, P. E. (2006) DNA secretion and gene-level selection in bacteria. *Microbiology* **152**, 2683-2688.
29. Spoering, A. L. & Gilmore, M. S. (2006) Quorum sensing and DNA release in bacterial biofilms. *Curr Opin Microbiol* **9**, 133-137.
30. van, d., V & Pretorius, P. J. (2008) Circulating DNA. Its origin and fluctuation. *Ann NY Acad Sci* **1137**, 18-26.
31. Uppuluri, P., Chaturvedi, A. K., Srinivasan, A. *et al.* (2010) Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog* **6**, e1000828.
32. Tamkovich, S. N., Vlassov, V. V., & Laktionov, P. P. (2008) Circulating deoxyribonucleic acids in blood and their using in medical diagnostics. *Mol Biol* **42**, 12-23.
33. Montagna, M. T., Caggiano, G., Borghi, E., & Morace, G. (2009) The role of the laboratory in the diagnosis of invasive candidiasis. *Drugs* **69**, 59-63.
34. Espinel-Ingroff, A. (2009) Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005-2009). *Rev Iberoam Micol* **26**, 15-22.
35. Kaplan, J. B. (2009) Therapeutic potential of biofilm-dispersing enzymes. *Int. J. Artif. Organs* **32**, 545-554.
36. Laniado-Laborin, R. & Cabrales-Vargas, M. N. (2009) Amphotericin B: side effects and toxicity. *Rev Iberoam Micol* **26**, 223-227.
37. Garcia-Effron, G., Chua, D. J., Tomada, J. R. *et al.* (2010) Novel FKS mutations associated with echinocandin resistance in *Candida* species. *Antimicrob Agents Chemother* **54**, 2225-2227.
38. Nett, J., Lincoln, L., Marchillo, K. *et al.* (2007) Putative role of β-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob Agents Chemother* **51**, 510-520.
39. Henriques, M., Martins, M., Azeredo, J., & Oliveira, R. (2007) Effect of farnesol on *Candida dubliniensis* morphogenesis. *Lett Appl Microbiol* **44**, 199-205.
40. Rossignol, T., Logue, M. E., Reynolds, K., Grenon, M., Lowndes, N. F., & Butler, G. (2007) Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob Agents Chemother* **51**, 2304-2312.
41. Weber, K., Schulz, B., & Ruhnke, M. (2010) The quorum-sensing molecule *E,E*-farnesol-its variable secretion and its impact on the growth and metabolism of *Candida* species. *Yeast* **in press**.
42. Langford, M. L., Atkin, A. L., & Nickerson, K. W. (2009) Cellular interactions of farnesol, a quorum-sensing molecule produced by *Candida albicans*. *Future Microbiol* **4**, 1353-1362.
43. Joo, J. H. & Jetten, A. M. (2010) Molecular mechanisms involved in farnesol-induced apoptosis. *Cancer Lett* **287**, 123-135.
44. Baldauf, S. L. & Palmer, J. D. (1993) Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. *Proc Natl Acad Sci USA* **90**, 11558-11562.
45. Davis-Hanna, A., Piispanen, A. E., Stateva, L. I., & Hogan, D. A. (2008) Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol Microbiol* **67**, 47-62.

46. Singh, A., Sharma, S., & Khuller, G. K. (2007) cAMP regulates vegetative growth and cell cycle in *Candida albicans*. *Mol Cell Biochem* **304**, 331-341.
47. Sullivan, D. J., Moran, G. P., & Coleman, D. C. (2005) *Candida dubliniensis*: Ten years on. *FEMS Microbiol Lett* **253**, 9-17.
48. Calderone, R. A. & Fonzi, W. A. (2001) Virulence factors of *Candida albicans*. *Trends in Microbiol* **9**, 327-335.
49. Frisvad, J. C., Andersen, B., & Thrane, U. (2008) The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. *Mycological Research* **112**, 231-240.
50. Lanças, F. M. (2003) The role of the separation sciences in the 21th century. *Journal of the Brazilian Chemical Society* **14**, 183-197.
51. Arthur, C. & Pawliszyn, J. (1990) Solid phase microextraction with thermal desorption using fused silica optical fibers. *Anal Chem* **62**, 2145-2148.
52. Solid phase microextraction: theory and optimization of conditions. (Bulletin 923). 1998. Sigma-Aldrich Co.
53. Gregus, P., Vlckova, H., Buchta, V., Kestranek, J., Krivcikova, L., & Novakova, L. (2010) Ultra high performance liquid chromatography tandem mass spectrometry analysis of quorum-sensing molecules of *Candida albicans*. *J Pharm Biomed Anal* **in press**
54. Winzer, K., Hardie, K. R., & Williams, P. (2002) Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **5**, 216-222.
55. Diggle, S. P., Gardner, A., West, S. A., & Griffin, A. S. (2007) Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? *Philos Trans R Soc Lond B Biol Sci* **362**, 1241-1249.
56. Biswas, S., Van, D. P., & Datta, A. (2007) Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* **71**, 348-376.
57. Calderone, R. A. (2002) Taxonomy and biology of *Candida.* In: Calderone, R. A. (ed) *Candida and Candidiasis*. Washington: ASM, pp 15-27.
58. Kuhn, D. M., Balkis, M., Chandra, J., Mukherjee, P. K., & Ghannoum, M. A. (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. *J Clin Microbiol* **41**, 506-508.
59. Lenz, A. P., Williamson, K. S., Pitts, B., Stewart, P. S., & Franklin, M. J. (2008) Localized gene expression in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **74**, 4463-4471.
60. Navarathna, D. H., Hornby, J. M., Krishnan, N., Parkhurst, A., Duhamel, G. E., & Nickerson, K. W. (2007) Effect of farnesol on a mouse model of systemic candidiasis: a DPP3 knock out mutant of *Candida albicans*. *Infect Immun* **75**, 1609-1618.
61. Hisajima, T., Maruyama, N., Tanabe, Y. *et al.* (2008) Protective effects of farnesol against oral candidiasis in mice. *Microbiol Immunol* **52**, 327-333.
62. Ricicova, M., Kucharikova, S., Tournu, H. *et al.* (2009) *Candida albicans* biofilm formaton in a new in vivo rat model. *Microbiology* **156**: 909-919.
63. Lazzell, A. L., Chaturvedi, A. K., Pierce, C. G., Prasad, D., Uppuluri, P., & Lopez-Ribot, J. L. (2009) Treatment and prevention of *Candida albicans* biofilms with caspofungin in a novel central venous catheter murine model of candidiasis. *J Antimicrob Chemother* **64**, 567-570.

64. Sugiura, Y. & Setou, M. (2010) Imaging mass spectrometry for visualization of drug and endogenous metabolite distribution: toward in situ pharmacometabolomes. *J Neuroimmune Pharmacol* **5**, 31-43.
65. Dickinson, J. R. (2008) Filament formation in *Saccharomyces cerevisiae* - a review. *Folia Microbiol (Praha)* **53**, 3-14.
66. Trofa, D., Gacser, A., & Nosanchuk, J. D. (2008) *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* **21**, 606-625.
67. Fidel, P. L., Jr., Vazquez, J. A., & Sobel, J. D. (1999) *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**, 80-96.
68. Horswill, A. R., Stoodley, P., Stewart, P. S., & Parsek, M. R. (2007) The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. *Anal Bioanal Chem* **387**, 371-380.
69. Dongari-Bagtzoglou, A., Kashleva, H., Dwivedi, P., Diaz, P., & Vasilakos, J. (2009) Characterization of mucosal *Candida albicans* biofilms. *PLoS One* **4**, e7967.

... "Cumpriu-se o mar..."

... "The sea is fulfilled..."

(In: "O profeta", Fernando Pessoa)